Journal of DENTAL RESEARCH

Founded by William J. Gies in 1919

Official Publication of the International Association for Dental Research

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Published for THE INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
By The C. V. Mosby Company, St. Louis 3, Mo.

Volume 29
1950

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Vol. 29 No. 1

Journal of DENTAL RESEARCH

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OFFICIAL PUBLICATION OF THE INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

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FEBRUARY, 1950

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Published for THE INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
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Vol. 29, No. 1. February, 1950. Journal of Dental Research is published bi-monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States, its Possessions, Pan-American Countries, $6.00 per volume; Canada and other foreign countries, $6.50. Single number when available, $1.25. Entered as second-class matter June 24, 1919, at the Post Office at St. Louis, Mo., under the Act of March 3, 1879. Printed in the U. S. A.

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THE EDITOR'S VIEWPOINT

Klinkenberg and Bibby (p. 4) have conducted an investigation long overdue. There was no actual evidence whether topically applied fluorides would or would not reduce caries activity in adults. Using 1 per cent fluoride solution after tooth cleaning and drying with alcohol and also using a 0.06 per cent solution of lead fluoride, they found 44.5 per cent and 27.5 per cent less new carious surface when compared with control quadrants. It is unfortunate that they varied the standard technic (of simply drying teeth before application) and strength of solution, because all that can be said is that sodium fluoride topically applied will reduce caries activity in young adults and to a greater extent than lead fluoride solution. However, to demonstrate that topically applied fluoride can reduce new cavity formation in young adults is a valuable contribution.

There are certain technical difficulties in the use of Hadley's tomato-juice agar medium for salivary lactobacillus counts. Efforts have been made to simplify plating and counting by various means. Diamond (p. 8) has added sodium azide to the standard medium and claims a marked increase in selectivity for lactobacilli. In all modifications, whether they be in technic or in collecting samples, plating material, type of media, method of counting, etc., clinical evaluations are necessary. What is a bacteriological "guesstimate," at its best, should not be changed without complete study. Diamond's proposal is worthy of clinical study.

Weisberger (p. 14) produced in vitro lesions in teeth that look convincingly like in vivo carious lesions. The carieslike lesions were produced over a period of time in saliva-glucose incubated media. Decalcified areas also were produced in media buffered to pH 4, without glucose, but they were not as much like carious lesions. No lesions occurred in the media without glucose added. Since these teeth were exposed to the low pH continuously for one week to four months the process is not identical with the intermittent process of caries in vivo. Perhaps lesions resembling the more chronic type of caries could be produced by exposing teeth to the media for short periods, daily, over longer periods of time. Many students of caries, from time to time, have stated that the lesions have not been produced in vivo. A study of Weisberger's illustrations and those in W. D. Miller's book should convince the open-minded.

Because dl-glyceric aldehyde had been shown to reduce acid formation in saliva during incubation, Shaw (p. 23) tried this compound in the food of white rats and cotton rats. The compound was used as dimer, while that tried in vitro may have been a monomer. The dl-glyceric aldehyde did not inhibit rat caries under the conditions of this study. Shaw calls attention to the fact that in vitro results should be further evaluated by in vivo studies. We should add that in vivo results in rats may not be comparable to results in man, as it has not yet been shown that rat caries and human caries are the same.

Hoppert and Shirley (p. 29) used radioactive phosphorus to aid in a search for any difference between the composition and metabolism of teeth from genetically caries-resistant and caries-susceptible rats. The only differences noted, in weight of teeth, per cent ash of molars, and per cent phosphorus of molars, were very slight. The search for the underlying factors within the teeth or their environment should be continued, as it offers a real clue to the etiological background of dental caries in rats.

Ludwick and Fosdick (p. 38) measured the ammonium content of the tooth brushings of 1,000 young men. They found no variation in the ammonium content of those who were clinically or bacteriologically caries immune or caries susceptible. A further investigation of the tooth scrapings, plus brushings, of 50 young men, showed no significant variation in ammonium content of immunes and susceptibles. Eighty per cent of the total mouth ammonia was removed by brushing alone. The possibility of high concentrations of ammonium being localized in very small areas to contribute to caries resistance is recognized by the authors.

Massler and MacDonald (p. 43) cultured hemolytic streptococci from the anterior gingivae of 6 per cent of 500 normal young adults while culturing the organism from 11.6 per cent of their throats. Only 11 individuals showed these streptococci in both the throat and gingiva. The danger of droplet infection of the dentist and auxilliary dental personnel should be evident from the percentage of "normal" individuals carrying the organism in their mouth and throats. The authors point out that the organisms can occur in the mouth without throat contamination and vice versa. They suggest, therefore, that in investigations of possible carriers both the mouth and throat be cultured.

Cohen and Fosdick (p. 48) have demonstrated that there is a decrease in gingival glycogen content of rabbits with alloxan diabetes. Earlier work from the same laboratory suggested that tissue glycogen may serve a protective function. On this basis, the authors propose that the increased susceptibility to periodontal disease in diabetes may be due to loss of gingival glycogen. Carrying their hypothesis further, they hint at the possible therapeutic effect of topically applied insulin, on the basis of a previously reported increase in gingival glycogen following topical application of insulin. The authors, rightly, warn that this is only a suggestion for future laboratory and clinical study.

Ostrom, Skillen, and Fosdick (p. 55) subjected the teeth of dogs to abnormal labial stress for two weeks and found no change in the gingival glycogen content. Histological changes, comparable to those seen in occlusal trauma, were observed. The authors conclude that "this study fails to support the hypothesis that occlusal trauma is a primary etiologic factor in periodontal disease." Likewise, it should be stated that it fails to support the concept that occlusal trauma is not a primary etiologic factor. The stress applied for two weeks is more comparable to that applied in splinting teeth for fractures and in orthodontia treatment than the chronic effect of occlusal disequilibrium that is considered a factor in periodontal disease. Chronic changes cannot be produced by rapid methods of investigation.

Perlman (p. 59) found that adding vegetable or mineral oil to the food of rats reduced the tendency toward cornification of the mandibular gingivae. He suggests that this is because the oils prevent dehydration. How this occurs in an area protected with the saliva is not explained, nor is the maxillary-mandibular variation explained. This same effect, i.e., protection by lubricating substances, should be considered in the use of a dry vs. a moist toothbrush.

Gurney and Huschart (p. 63) found that the ingestion of water had practically no effect on salivary phosphorus or calcium concentration and that the ingestion of heavy concentrates of dicalcium phosphate increased salivary phosphorus but did not regularly increase salivary calcium. They suggest that the relationship between actual and critical pH is of more importance in calculus formation than Ca/P content of saliva.

Harris and Brandel (p. 68) used electric current through the dental pulp to measure analgesia. They found that aspirin, codeine sulphate and placebos gave significantly higher threshold of pain than the current without medication. They do not seem to believe that the comparative results with a placebo and possibly analgesic drugs are untenable, as suggestion can reduce painful response. One possible improvement would be to drill a small hole, the size of the electrode, and to insert the electrode in this same hole for each reading.

Hunter (p. 73) investigated the effect of amino acid deficiency on the growth of rat teeth and on their other organs. He found that the rats lost weight and showed tissue atrophy but that the histodifferentiation of the teeth was normal. The quality was maintained even though the quantity was reduced. The property of the dental tissues to claim a high priority on meager supplies of essential amino acids is suggestive of their same ability with calcium.

Irving (p. 87) has examined sections of teeth from normal, rachitic, and treated rachitic rats after staining with silver salts. Irving points out that many details brought out by the silver stains were not observed under hematoxylin and eosin and vice versa. It could be added that in the study of normal and experimental material it is wise to utilize various staining and preparation technics for each may emphasize details that are obscure in the other methods. Certainly this is espe-

cially true of ground sections and decalcified sections. In rickets, Irving observed delay in calcification of the differentiated dentin. In healing, accelerated calcification of the cementing substance and reduced fibril formation was noted.

English and Dudley (p. 93) injected radioactive gallium lactate into rats and rabbits and observed radioautographs of their teeth and jaws. The methods and illustrations are interesting. The authors point out the advantages of radioautographs over Geiger counts. Apparently gallium plays no part in the metabolism of enamel and dentin.

Di Salvo and Neumann (p. 101) fed two groups of five monkeys, each, on practically the same diet, except that the food of one group was soft and that of the other required vigorous mastication. The molars of those fed the soft diet had a water content 10 per cent higher than those of the vigorously chewing animals. Some years ago a higher water content was demonstrated in sound tissues from carious teeth than that from noncarious teeth (Crowell, Hodge, and Line). The relationship of these two separate findings remain for further investigation.

H. B. G. R.

THE EFFECT OF TOPICAL APPLICATIONS OF FLUORIDES ON DENTAL CARIES IN YOUNG ADULTS

EGIL KLINKENBERG AND B. G. BIBBY*

Tufts College Dental School, Boston, Mass.

BECAUSE it has not yet been demonstrated that topical applications of sodium fluoride reduce dental caries in adults, it has commonly been assumed that fluoride applications are effective only in children. This idea has been strengthened by the failure of experiments using a fluoride dentifrice,[1] a fluoride mouthwash,[2] and a single application of an acid fluoride solution[3] to give caries reductions in young adults. Some theoretical considerations[4] also might suggest that fluorides might react less actively with mature than with newly erupted teeth. However, until clinical tests demonstrate that a treatment program which is effective in children is ineffective in adults, the conclusion that topical fluoride treatments do not prevent caries in adults is not warranted. This paper reports the effects on adults of a test of the sort which has repeatedly produced caries reduction in children.

DESCRIPTION OF STUDY

In October and November, 1946, the sophomore and freshman students at Tufts College Dental School were given careful clinical examinations using full-mouth and bite-wing x-rays. All fillings, cavities, and missing teeth were recorded on suitable charts while the student was under examination in the dental chair. When this initial examination was made the age range of the students was 18 to 40 years (average 25 years).

At the time of the first examination and at approximately three monthly intervals thereafter, single fluoride treatments were given to the teeth of each student. Each treatment consisted of cleaning all the teeth with a rubber cup using pumice and water, making no effort to polish the teeth or give a classical prophylaxis. Thereafter, one quadrant was isolated with cotton rolls, the teeth dehydrated with alcohol and air and the fluoride solution applied. In making the fluoride applications, the dried quadrants were thoroughly wetted by means of cotton pellets soaked in the fluoride solution, taking care that the solution penetrated into the interproximal spaces and the fissures on the occlusal surfaces. After letting the solution remain on the surface for a short period, the application was repeated. An average of about five minutes was required for the treatment of each quadrant. Upper and lower quadrants were treated alternately with a 1.0 per cent solution of sodium fluoride and an 0.06 per cent solution of lead fluoride. The two remaining quadrants in each mouth were not treated and were left to serve as controls. Because of convenience of access, most of the fluoride applications were made on the left quadrants but right quadrants were treated sufficiently often to discourage the examiner from interpreting the results in relation to a known treatment program.

*Present Address, Eastman Dental Dispensary, Rochester, N. Y.

Received for publication, March 30, 1949.

After a period of approximately fourteen months, the teeth were re-examined with new x-rays. Actually, a few of the students were examined within twelve months and some after fifteen months, but for the bulk of them the study period was fourteen months.

RESULTS

Out of a total of 150 students treated at the beginning of the study, only 139 completed the treatment routine satisfactorily and were available for re-examination. Of these, 10 received only three treatments instead of the four given to the remainder of the group. The effect on caries incidence of the topical treatments with sodium fluoride is presented in Table I. This table shows the number of caries-free surfaces in the treated and control groups at the beginning and the end of the treatment period. The difference between these represents the number of new D.M.F. surfaces appearing during the treatment program. It can be seen that there is about 44.5 per cent reduction in the number of new D.M.F. surfaces in the treated quadrants as compared with the control quadrants. It should be noted that the figure for new D.M.F. surfaces includes not only new carious surfaces, but also sound surfaces which had been included in fillings or lost as a result of extraction of a tooth. Because of the completeness of the student records and the availability of the operator and student for re-examination and consultation, it is possible by excluding from the D.M.F. figures those surfaces which had been added for the reasons given above to establish corrected figures which represent the actual number of new carious surfaces. These figures are compared in the last column of the table. The corrected figures reveal an even larger difference (56.6 per cent) between the amount of new caries in the treated and the untreated quadrants. If the findings are presented in terms of D.M.F. teeth, the reduction in caries incidence is only 27 per cent. This figure is, however, even less reliable than that for the D.M.F. surfaces because in our student group the teeth had already been exposed to the vicissitudes of dental school existence and were heavily filled at the beginning of the study. As a result the initial number of sound teeth was inordinately low totaling only 314 for the control and 341 for the test quadrants. Of these 29 and 23, respectively, showed new decay.

TABLE I

CARIES INCREMENT IN SODIUM FLUORIDE—TREATED AND CONTROL QUADRANTS OF 139* ADULT PATIENTS

QUADRANTS	NO. OF CARIES-FREE SURFACES		NEW D.M.F. SURFACES	NEW CARIOUS SURFACES†
	FIRST EXAM.	SECOND EXAM.		
NaF treated	3141	3044	97	66
Untreated control	3079	2906	173	152
Difference			44.5%	56.6%

*Includes 10 patients who received only three treatments.
†Excludes initial caries-free surfaces included in restorations or extracted teeth.

In Table II comparable data are presented for the quadrants treated with lead fluoride. With this treatment, caries reductions of 27.5 per cent in new

TABLE II

CARIES INCREMENT IN LEAD FLUORIDE—TREATED AND CONTROL QUADRANTS OF 139* ADULT PATIENTS

QUADRANT	NO. OF CARIES-FREE SURFACES		NEW D.M.F. SURFACES	NEW CARIOUS SURFACES†
	FIRST EXAM.	SECOND EXAM.		
PbF_2 treated	3174	3053	121	80
Untreated control	3207	3040	167	130
Difference			27.5%	38.5%

*Includes 10 patients who received only three treatments.
†Excludes initial caries-free surfaces included in restorations or extracted teeth.

D.M.F. surfaces and 38.5 per cent in new carious surfaces are observed. In terms of D.M.F. teeth the reduction was 32 per cent.

If only the 129 students who received the four topical treatments are considered, we find a reduction in new carious surfaces of 47.3 with sodium fluoride applications and 39.6 with lead fluoride applications. It is interesting to notice that both for the sodium fluoride- and lead fluoride-treated quadrants there is, as in other studies of this sort, a greater caries reduction in upper than in the lower teeth. In this study sodium fluoride gave a 58 per cent reduction of new caries in the upper teeth and 39 per cent in the lower teeth. Lead fluoride produced reductions of 45.3 per cent and 31.3 per cent, respectively. It was also found that approximately 13 per cent, or 18 of the 129 students, given four treatments, showed more caries in the treated than in the control quadrants when treated with sodium fluoride, and 18 per cent, or 23 students, revealed more caries in the treated quadrants in the lead fluoride program. Only 2 students had more caries on both the lead fluoride- and sodium fluoride-treated sides. While the figures are too small to have any significance. it may be of some interest to record that 22 instances of arrested or inactive caries were found in the sodium fluoride-treated quadrants, as compared with 10 in the control quadrants. No difference in the amount of arrested caries appeared between the quadrants treated with lead fluoride and the control quadrants.

TABLE III

COMPARISON OF CARIES INCREMENT IN UPPER AND LOWER TEETH OF 129 ADULT PATIENTS

TREATMENT	TEETH	NEW CARIOUS AREAS		
		TREATED QUADRANTS	CONTROL QUADRANTS	DIFFERENCE (PER CENT)
Sodium fluoride				
	Upper	40	89	55.0
	Lower	50	82	39.0
	Both	90	171	47.3
Lead fluoride				
	Upper	52	95	45.3
	Lower	44	64	31.3
	Both	96	159	39.6

COMMENT

The fact that a definite reduction in dental caries seems to be demonstrated in this study is somewhat out of line with existing concepts that sodium fluoride applications have no effect on dental caries in adults. If, however, the

evidence from which such conclusions were drawn is examined, our positive results are not surprising. For instance, our failure to demonstrate a caries reduction as the result of using a sodium fluoride mouthwash[2] or dentifrice[1] in a comparable student group can be explained just as well as the result of the ineffectiveness of the methods of application used (dentifrice and mouthwash) or of the particular fluoride mixtures used in those studies. The failure of Arnold, Dean, and Singleton[3] to produce a caries reduction when they made a single application of a one per cent acidulated sodium fluoride to the mouths of a group of young adults, aged 18 to 22, may be accounted for either on the basis of the single application, which Galagan and Knutson[5] have shown to be less effective than more frequent applications, or on the basis of the use of an acidulated fluoride solution which has since, both in human[6] and animal experiments,[7] proved to have no caries-inhibiting effect. In short, there is nothing in the published literature on the effect of sodium fluoride on caries activity which is really contradictory to our findings.

The higher caries reductions shown in this study as compared with those using other procedures, despite the possible disadvantage of a spaced treatment program[5] and a weaker fluoride solution, suggest that the most commonly advocated procedure for giving topical fluoride treatments for caries control may be a long way from the most effective possible method. The procedures now used have been arrived at purely empirically and are based on somewhat crude clinical experimentation. It is possible that the careful technics of dehydration or application used by us may have special significance. It would probably be well if more careful attention were paid to these points in giving fluoride treatments, and if it were kept in mind that there is need for much further experimentation to determine whether additional cleanings, the use of mucin solvents, chemical dehydration, wetting agents, or more careful technics of application might not increase the effectiveness of fluoride therapy.

SUMMARY

At approximately three monthly intervals single quadrants of the teeth of 139 students, aged 18 to 40 years, were treated with solutions of 1.0 per cent sodium fluoride of 0.06 per cent lead fluoride.

After approximately fourteen months, the quadrants treated with sodium fluoride showed 44.5 per cent less new D.M.F surfaces, or 56.6 per cent less new carious surfaces, than did the untreated control quadrants on the opposite sides of the mouths.

The quadrants treated with lead fluoride showed 27.5 per cent less new D.M.F. surfaces, or 38.5 per cent less new carious surfaces, than their control quadrants.

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A SELECTIVE MEDIUM FOR LACTOBACILLI COUNTS FROM SALIVA

BEN ELKAN DIAMOND, B.S., M.A.

With the Technical Assistance of Audrey Walstad, B.S.

Division of Laboratories, State Board of Health, Pierre, South Dakota

IN DECEMBER, 1947, the Division of Dental Health requested this laboratory to undertake lactobacilli counts from saliva specimens. These specimens were to be submitted by the dentists in South Dakota. It became evident immediately that overgrowth by other bacteria, yeasts, and, in a few instances, molds would make it impossible to report satisfactorily the results of many specimens. Such unsatisfactory plates approximated 20 per cent of the total examined. The medium specified (tomato-juice agar[1]) exerts some bacteriostatis on other bacteria than the lactobacilli because of its acidity, pH 5.0. Many genera of bacteria and related organisms are not inhibited however.

Before an attempt was made to develop a selective medium, appropriate tests were run to eliminate the possibility of contamination in the laboratory. Such contamination was possible because of exceptionally crowded conditions. Tests on all the pertinent material indicated adequate sterility. Uncovered plates were exposed for periods up to five minutes and then incubated. Negligible counts resulted. The organisms responsible for unsatisfactory plates were present in the saliva.

Media which will select and/or differentiate bacteria are invaluable where mixed flora are encountered. In the former, the selective action is generally induced with chemical compounds or dyes. These media usually select out broad groups of bacteria; the gram-negative, gram-positive, or genera within those groups. The differential media employ suitable means of indicating biochemical activity and are useful in differentiating species, for example, lactose fermenters from nonfermenters, etc. Both may be combined to devise a selective and differential medium.

Hartman[2] reported the selective action of sodium azide in 1936. Mallman[3] utilized this compound to suppress colon bacilli in water specimens. Snyder and Lichenstein[4] reported the suppression of gram-negative bacteria with sodium azide. Mallman, Botwright, and Churchill[5] included this compound in their study of the bacteriostatic effect of slow-oxidizing agents. The results obtained in this investigation were the basis for the previous paper by Mallman.[3] One of us[6] in an investigation of the tolerance of diphtheria bacilli to sodium azide noticed the suppression of many of the oral bacteria present on Loeffler slants inoculated for the diagnosis of diphtheria. Sodium azide seemed to be a suitable agent to investigate for the purpose of preparing a selective medium for the enumeration of lactobacilli.

Received for publication, April 11, 1949.

PROCEDURE

Appropriate amounts of sodium azide* were added to the basic tomato-juice medium[1] either by dilution from a 1:100 stock solution or by weight. The final dilutions of the reagent in the medium were 1:1000, 1:2500, 1:5000, 1:7500, 1:10,000, and 1:15,000. The stock 1 per cent solution was not sterilized as in this concentration sodium azide is highly bacteriostatic. The reagent is stable to autoclave sterilization, however. Preliminary experiments were made to determine the optimum level of sodium azide which would suppress the maximum of contaminants with as little as possible deleterious effect on the lactobacilli. The technic of the counts utilizes a broth tube as well as a plate in order to determine counts lower than 50 per milliliter. When excessive overgrowth appeared on a plate, the tube from this specimen was plated on sodium azide tomato-juice agar utilizing the dilutions mentioned. In addition pure cultures of lactobacilli and contaminating bacteria isolated from the original plates were tested against similar dilutions.

When the critical levels of sodium azide were determined, it was incorporated into the basic medium and specimens submitted to the laboratory were plated in duplicate utilizing one plate with the chemical and one without.

RESULTS

In plating growth from the broth tubes and also in pure culture study, it became apparent that it was possible to inhibit a wide variety of bacteria, yeasts, and molds at azide dilutions of 1:7500 and 1:10,000 without any obvious toxicity to the lactobacilli. Most strains of micrococci, staphylococci, streptococci, molds, and yeasts in addition to the gram-negative Aerobacter and Proteus species were markedly inhibited. The inhibitory effect to the gram-positive bacteria is probably a combination of chemical bacteriostasis and pH.

The results of the duplicate platings are recorded in Table I. Two pairs of the plates are shown in Figs. 1 and 2. The first shows colonies which, on visual examination, are difficult to distinguish from the rough lactobacillus. Fig. 3 is the photomicrograph and an obvious streptococci is responsible. This and the yeast shown in Fig. 2 are completely suppressed by the sodium azide.

DISCUSSION

Beginning on May 1, 1948, each saliva sample received in this laboratory was plated in duplicate on conventional Hadley tomato agar[1] and on Hadley tomato agar to which sodium azide had been added to bring the azide concentration to 1:10,000. This series included 476 consecutive specimens. Of the Hadley plates, 131 (27.5 per cent) were uncountable because of overgrowths, mostly of yeasts and molds. Four of the azide plates (0.85 per cent) were likewise overgrown. Remaining were 345 duplicate plates on which comparison counts could be made. Results of this count comparison are shown in Table I.

The mean counts in all categories were very much alike, and in 156 of the cases (45 per cent) the counts were virtually identical. But the ultimate test

*Practical grade—Eastman Kodak Company, Rochester, N. Y.

TABLE I

LACTOBACILLUS COUNTS IN A SERIES OF 345 CONSECUTIVE SALIVA SAMPLES PLATED IN DUPLICATE ON HADLEY TOMATO AGAR AND ON HADLEY TOMATO AGAR PLUS 1:10,000 SODIUM AZIDE (NaN_3)

RANGE OF HADLEY COUNT	NUMBER OF COMPARABLE PLATES	MEAN COUNTS		DEVIATION IN AZIDE PLATES					NUMBER OF CASES IN WHICH CLINICAL COURSE WOULD DIFFER
				HIGHER COUNTS		SAME COUNT			
		HADLEY PLATES	AZIDE PLATES	NUMBER OF PLATES	MEAN DEVIATION		NUMBER OF PLATES	MEAN DEVIATION	
0	46	0	9	5	+80	41	0	--	0
1 to 299	14	122	132	3	+250	5	6	-103	0
300 to 999	16	500	420	7	+100	2	7	-285	1
1,000 to 9,999	67	4,700	4,800	24	+1,600	15	28	-1,000	2
10,000 to 49,999	60	31,300	29,000	9	+13,500	24	27	-4,200	5
50,000 and more	142	316,000	306,000	25	+37,800	69	48	-49,000	6
Total	345	136,500	132,000	73	+15,100	156	116	-21,500	14

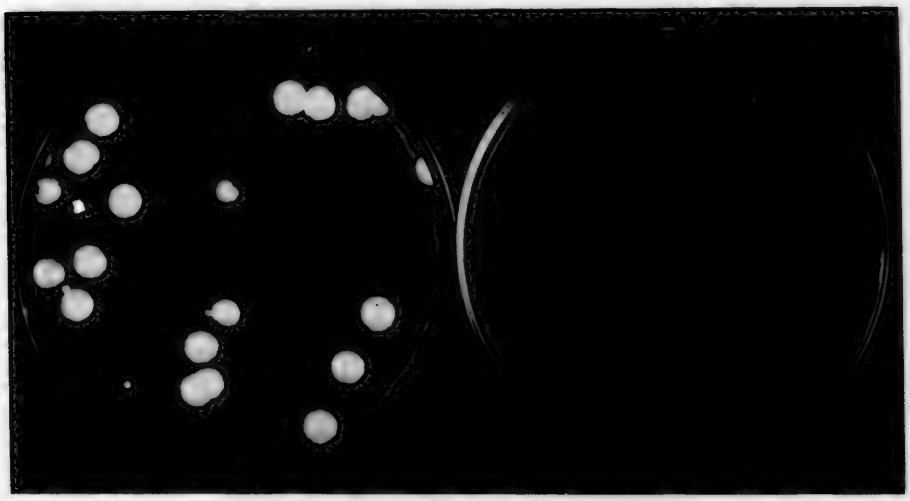

Fig. 1.—Duplicate plating showing inhibition of yeast and streptococci. Sodium azide plate on right. (×1.0)

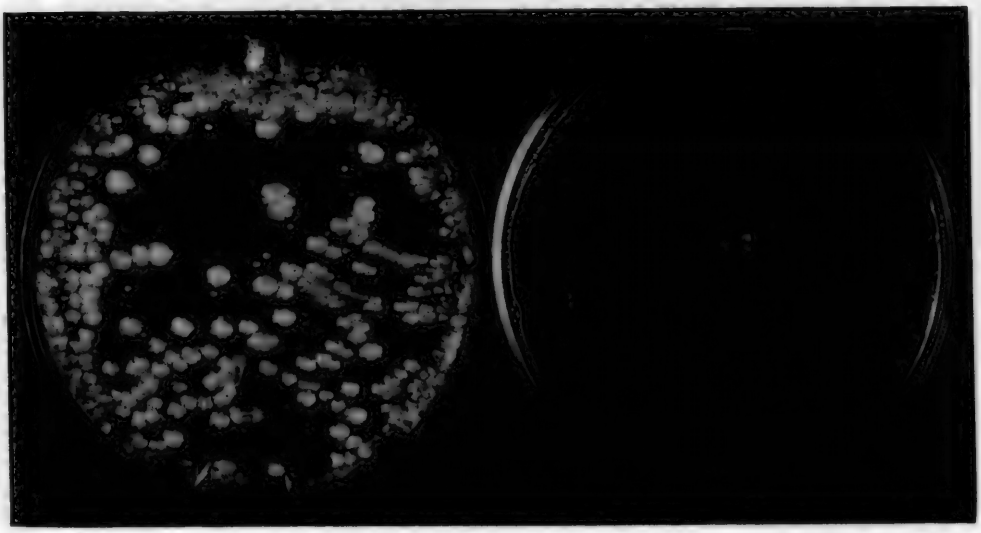

Fig. 2.—Duplicate plating showing effect of sodium azide 1:10,000 in suppression of yeasts. Right-hand plate is pure culture of lactobacilli. (×1.0)

of any laboratory procedure is its clinical utility. Except for 14 cases the clinical indications of the counts were the same.

In 13 of these 14 cases the azide count was materially lower than the Hadley count. This may have been due in part to an error in which a certain streptobacillus was counted as a lactobacillus on the Hadley plates, particularly early in the plates. This streptobacillus grows in a rough colony very similar to one

of the rough forms assumed by lactobacilli on the Hadley plate; it is inhibited on the azide plate. It seems probable that count deviations might have been random if this contaminant had not been included as a lactobacillus in many counts of the Hadley plates. It appears, therefore, that the selective medium presented for the enumeration of lactobacilli in saliva specimens have distinct advantages over the Hadley plate. Plates may be counted without interference from contaminants. It is possible to report the results of every specimen and dentists are no longer confused with an "unsatisfactory plate" report. There is a saving in the time necessary to train an individual to this procedure in addition to reducing the number of selections of colonies for identification purposes.

Fig. 3.—Photomicrograph of colony shown in Fig. 1. (×900)

The Hadley plate with sodium azide is now employed routinely for lactobacilli determinations. The azide may also be added directly to the medium and sterilized. Such plates are stable for periods up to three weeks if stored in the refrigerator to prevent drying. The 1 per cent stock azide solution loses its bacteriostatic activity after six weeks' storage so it is best to prepare this in small amounts where it is added to the sterilized medium and not previous to sterilization.

SUMMARY

The results of a selective medium based on sodium azide is presented. This selective medium is sodium azide 1:10,000 in Hadley tomato agar.[1] Excellent inhibition of contaminants present in saliva is obtained with little clinically significant alteration of the lactobacilli count.

Appreciation is expressed to A. L. Russell, D.D.S., Director, Division of Dental Health, for the presentation of the data in Table I and its analysis, and to C. O. Vickmark, Law Enforcement Division, Attorney General's Office, for the photographs.

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A ROLE OF GLUCOSE IN THE PRODUCTION OF ARTIFICIAL CARIES

DAVID WEISBERGER, D.M.D., M.D.

Harvard School of Dental Medicine, Boston, Mass.

INTRODUCTION

OBSERVATIONS on artificial caries have been reported by many workers.[1-6] Most of these reports have been concerned with the effect of exposing teeth to growing bacteria. Our interest has been focused on nutritional requirements for oral bacteria.[7] We have also observed that when glucose is added to saliva a variety of chemical changes occur during incubation; of particular interest to us is an increase in a-amino acids.[8] Under these experimental conditions saliva can be used to supply the a-amino acid nutriments for growth.[9] Because of our continued interest in these factors associated with bacterial growth, it seemed important to study the effect of exposing intact teeth to the action of mixed oral flora incubated in a medium in which the nutriments for growth were mostly known chemical substances. It was hoped that in following the chemical changes occurring in the nutrient medium some information could be obtained regarding the biochemical processes associated with dental caries.

EXPERIMENTAL

Treatment of Teeth.—Unerupted, intact teeth were used for these experiments. Each tooth was covered with sticky wax with the exception of a small area on one of the smooth surfaces of the crown. X-ray examination was then made taking two views, one with the exposed surface in contact with the film and the other a lateral view of the exposed surface. This was done for the purpose of confirming the intact appearance of the enamel. Kodachrome pictures were also taken of the exposed surface. The teeth were suspended in the inoculated medium and were incubated at 37.5° Centigrade. The medium and the inoculum were changed at weekly intervals for a period of four months. Chemical determinations were made immediately after the change of medium and inoculum and also after 48 and 168 hours of incubation. The chemical changes reported for each experiment were found to be consistent throughout the incubation period. During the period frequent x-ray and photographic examinations were made.

Presented at a meeting of the Boston Section of International Association for Dental Research, Feb. 17, 1948.

Received for publication, March 19, 1949.

Preparation of Basic Medium.—It was found that the following medium gave luxuriant growth of the organisms:

12.5 ml. casein hydrolyzate
12.5 ml. liver extract*
1.5 Gm. sodium acetate
1.25 ml. salt solution A†
1.25 ml. salt solution B‡
2.5 Gm. glucose

This was made to a volume of 250 ml. with distilled water. The medium was then sterilized in 60 ml. aliquots by autoclaving at 10 pounds' pressure for 10 minutes. These aliquots were then used in the experimental work.

Preparation and Collection of Inoculum.—Paraffiin-stimulated saliva was collected from 6 individuals and pooled. This was used in the preparation of the inoculum. Fifteen milliliters of saliva were centrifuged, the supernatant poured off, and the sediment made up to a volume of 3 ml. with sterile saline. One milliliter of this suspension was used as the inoculum for 60 ml. of medium.

Chemical Methods.—Analysis for free amino acids was by the gasometric method of Van Slyke, Dillon, MacFadyen, and Hamilton.[10] Ammonia (plus urea) nitrogen was determined by the hypobromite method of Van Slyke.[11] pH determinations were made with the glass electrode of a Coleman pH electrometer. Reducing substance was determined by the method of Shaffer and Somogyi.[12] Lactic acid was determined colorimetrically by the method of Barker and Summerson.[13] Calcium was determined by the method of Kramer and Tisdall.[14]

RESULTS

Effect Without Glucose.—The data for Tooth 401 B in Table I are typical of experiments in which the basic medium as described above was used without the added glucose. By means of the photographic and x-ray examinations mentioned above, we were unable to demonstrate any changes in the exposed tooth surface after four months of incubation (Fig. 1). The chemical changes occurring in this medium during incubation are characteristic of changes occurring in a medium without glucose. As shown in Table I the pH rose sharply from 5.95 at 0 time to a pH of 8.6 at 168 hours. The increase in ammonia (plus urea) nitrogen and the decrease in a-amino acids are evidence of a rapidly deaminating process. No lactic acid was formed and none was expected since there was no glucose added to the medium. Although no carbohydrate was added there is a small amount of reducing substance present. There was no increase in calcium in the medium.

*The crude liver extract was used primarily as a source of the water soluble vitamins. Preparation of liver extract: 8.5 ml. of crude Lilly Liver Extract (2 injectable U.S.P. units per ml.) were diluted to 100 ml. with sterile distilled water.

†*Salt Solution A*

K_2HPO_4	5.0	Gm.
KH_2PO_4	5.0	Gm.
Dist. H_2O	50.0	ml.

‡*Salt Solution B*

$MgSO_4.7H_2O$	10.0	Gm.
NaCl	0.5	Gm.
$FeSO_4.7H_2O$	0.5	Gm.
$MnSO_4.2H_2O$	0.337	Gm.
Dist. H_2O	250.0	ml.

TABLE I
EFFECT OF BASIC MEDIUM WITHOUT GLUCOSE AND WITHOUT BUFFER

	TOOTH 401 B		
	0 TIME	24 HOURS' INCUBATION	168 HOURS' INCUBATION
pH	5.95	8.00	8.60
Reducing substance mg./ml.	0.10	0.04	0.03
Lactic acid mg./ml.	0.03	0.02	0.01
Calcium mg./ml.	0.01	0.01	0.01
Ammonia and urea nitrogen mg./ml.	0.32	1.11	0.90
A-amino nitrogen mg./ml.	0.598	0.094	0.088

Effect With Glucose.—The data for Tooth 400 B in Table II were taken from an experiment typical of those in which the tooth was incubated in a medium identical to that used in the previous experiment except for the addition of 1 per cent glucose. An aliquot of the same inoculum was used. After one to two weeks of incubation a brown pigmentation occurs on the exposed tooth surface. X-ray examination shows a beginning rarefaction in the enamel, progressing toward the dentin. After four months' incubation the pigmented area becomes darker and involves the entire exposed surface. X-ray examination at this time reveals marked demineralization of the enamel

TABLE II
EFFECT OF BASIC MEDIUM WITH GLUCOSE AND WITHOUT BUFFER

	TOOTH 400 B		
	0 TIME	24 HOURS' INCUBATION	168 HOURS' INCUBATION
pH	5.95	4.15	4.25
Reducing substance mg./ml.	9.60	0.19	0.10
Lactic acid mg./ml.	0.06	4.60	5.00
Calcium mg./ml.	0.01	0.11	0.15
Ammonia and urea nitrogen mg./ml.	0.32	0.35	0.41
A-amino nitrogen mg./ml.	0.634	0.569	0.542

and dentin (Fig. 2). Histological examinations of ground sections after four months of continuous incubation revealed a dark brown pigmented carious matrix with surface continuity in that portion of the enamel which was exposed to the medium. At the dentino-enamel area the dentinal tubules were swollen, irregular, and showed evidence of destruction (Fig. 3). Some decalcified sections were studied by means of Gram stain and revealed spheroid gram-positive bodies in the dentinal tubules.

With glucose present in the medium the chemical changes are quite different from the previous experiment. Lactic acid is formed in a considerable quantity and associated with this there is a depression of the pH to 4.1. The increase in calcium in the medium is consistent with the x-ray evidence of demineralization of the tooth enamel. Of particular interest is the slow rate

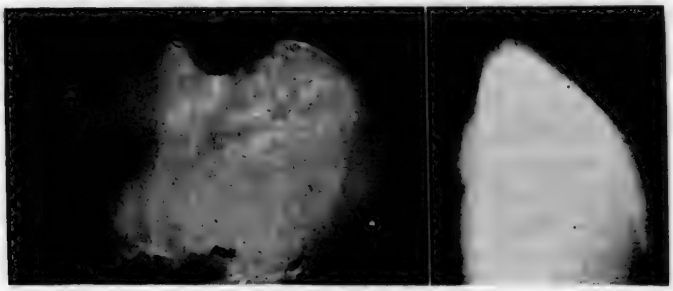

Fig. 1.—Tooth 401 B (no glucose).
Left: Intact exposed surface after four months' incubation.
Right: X-ray lateral view of exposed surface showing no demineralization after four months' incubation.

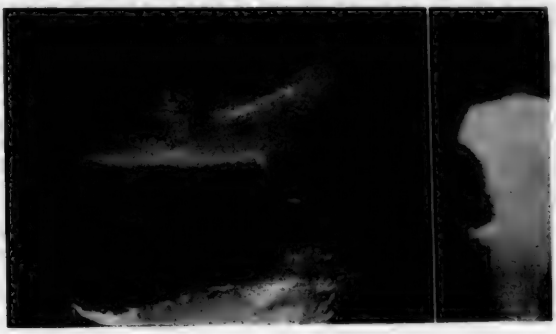

Fig. 2.—Tooth 400 B (glucose present).
Left: Dark area on exposed surface.
Right: X-ray lateral view of exposed surface after four months' incubation.

Fig. 3.—Tooth 400 B (glucose present).
Left: Low-power ground section showing dark pigmented area in enamel and involvement of dentin in carious process.
Right: High-power ground section showing swollen dentinal tubules at area of carious enamel matrix.

at which the a-amino acids disappear when glucose is present in the medium. The very minimal rise in ammonia (plus urea) nitrogen suggests a decreased rate of deamination of the a-amino acids. The glucose disappears at a rapid rate so that at 24 hours there is less than one half of the original concentration and at 48 hours there is practically no glucose remaining. The deamination of the a-amino acids, however, continues at a very slow rate.

Effect With pH 4.0 Buffer Without Glucose.—In preliminary experiments it was found that when the medium was maintained at a pH 4.0 by the addition of a buffer* without the addition of glucose, the change in the a-amino acid concentration during incubation as well as the ammonia (plus urea) nitrogen was similar to that observed when the medium was incubated with 1 per cent glucose. Because of this similar effect on the nitrogen changes an experiment was made to test a medium without glucose, but maintained at a pH of 4.0 for its ability to produce a carious-like lesion in an intact tooth. The data for Tooth 401 A in Table III are typical of experiments in which the basic medium without glucose, but maintained at a pH of 4.0 with added buffer, was used. (pH 4.0 was chosen as it is the H-ion concentration observed in the incubating medium when glucose is present.) After three weeks' incubation a change occurred on the exposed tooth surface. This change consisted primarily of a soft amorphous decalcification without any brown pigmentation (Fig. 4). This white chalkiness washes out readily and resembles the change occurring in teeth which are undergoing decalcification preparatory to histological study. The significant chemical changes in the medium were a definite increase in the calcium and the almost complete maintenance of the original a-amino acid concentration with minimal ammonia formation.

TABLE III

EFFECT OF BASIC MEDIUM WITHOUT GLUCOSE AND WITH pH 4.0 BUFFER

	TOOTH 401 A		
	0 TIME	24 HOURS' INCUBATION	168 HOURS' INCUBATION
pH	4.00	4.00	4.27
Reducing substance mg./ml.	0.16	0.14	0.12
Lactic acid mg./ml.	0.02	0.03	0.13
Calcium mg./ml.			
Ammonia and urea nitrogen mg./ml.	0.01	0.08	0.35
	0.32	0.38	0.30
A-amino nitrogen mg./ml.	0.610	0.577	0.444

Effect With pH 8.0 Buffer With Glucose.—Having just demonstrated that the effect of glucose on the production of the carious-like lesion is only partially due to a lowered pH (Tables I and III) we attempted to control the fall in pH consistently observed during incubation when glucose is present. This was done by making up the basic medium with 1 per cent glucose and maintaining the pH at 8.0 by means of a buffer.† (This pH was chosen as it is the H-ion concentration observed in an incubating meduim in which there

*Citric acid and Na_2HPO_4.

†KH_2PO_4 and NaOH.

is no glucose.) The data for Tooth 400 A in Table IV were taken from an experiment typical of those in which the tooth was incubated in the basic medium with 1 per cent glucose and containing pH 8.0 buffer. No change was observed on the exposed tooth surface, nor was there any x-ray change noted (Fig. 5). The carbohydrate in the medium was utilized and lactic acid was formed at 48 hours, but when determinations were made after 168 hours of incubation, there was only a trace of lactic acid present. This rapid disappearance of lactic acid between a pH of 7.0 and 8.0 is in accord with the findings of Muntz.[15] No depression of pH occurred. Apparently at a pH of 8.0, rapid deamination of the a-amino acids occurs regardless of the presence of glucose. It is of interest to note that although glucose is present in the medium and is utilized, it does not contribute to the development of the carious-like lesion when the acid formed is buffered at a pH of 8.0.

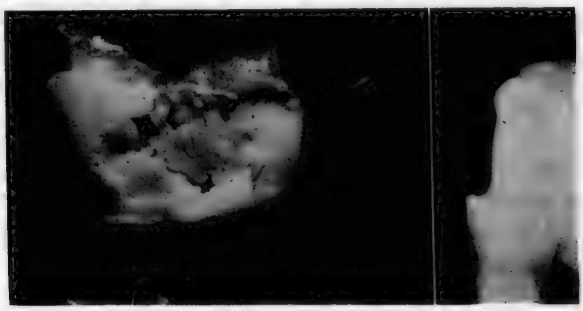

Fig. 4.—Tooth 401 A (no glucose, buffered at pH 4.0).
Left: White amorphous decalcification on exposed surface.
Right: X-ray lateral view showing decalcification after four months' incubation.

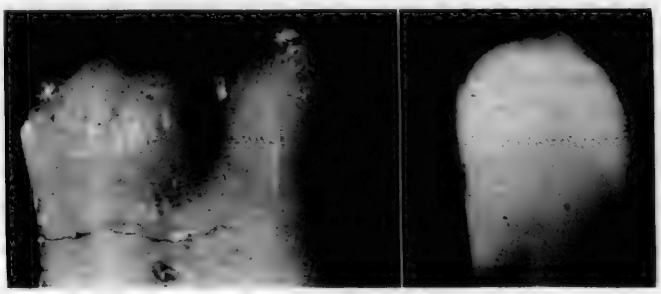

Fig. 5.—Tooth 400 A (glucose present, buffered at pH 8.0).
Left: Intact exposed surface.
Right: X-ray lateral view showing no demineralization.

DISCUSSION

It appears from the above experiments that no decalcification of tooth enamel occurs in an environment of bacterial activity at a pH of 8.0, attained either as the result of metabolizing bacteria in the absence of carbohydrate or by maintenance of a pH of 8.0 by the addition of buffer to the medium in the presence of carbohydrate. This, however, can not be attributed to the

TABLE IV
EFFECT OF BASIC MEDIUM WITH GLUCOSE AND PH 8.0 BUFFER

	TOOTH 400 A		
	0 TIME	24 HOURS' INCUBATION	168 HOURS' INCUBATION
pH	8.00	8.00	8.77
Reducing substance mg./ml.	7.80	0.81	0.46
Lactic acid mg./ml.	0.21	2.90	0.25
Calcium mg./ml.	0.01	0.01	0.01
Ammonia and urea nitrogen mg./ml.	0.32	0.36	0.58
A-amino nitrogen mg./ml.	0.634	0.390	0.100

cessation of bacterial activity because the inoculated medium in which the tooth was suspended was changed weekly. Repeated chemical determinations following the changes in medium and inoculum have demonstrated that the tooth was exposed to a continual environment of bacterial activity. In contrast to this are the experiments in which decalcification of tooth enamel occurs in an environment of bacterial activity at a pH of 4.0 attained by the formation of lactic acid from carbohydrate breakdown or maintained at a pH of 4.0 by the addition of buffer in the absence of carbohydrate.

Therefore, it seems from these experiments that a low pH is necessary for decalcification of tooth enamel. This pH can be attained in vitro by supplying suitable nutriments for bacterial growth and a carbohydrate as a substrate for acid formation. In comparing the gross and textural differences of the teeth in which decalcification occurred, the tooth in contact with an environment maintained at a pH 4.0 without glucose developed a jelly-like white decalcified area which offered no resistance to an explorer and was incapable of fixation for histological study. In the tooth exposed to an environment in which a pH of 4.0 was attained by means of carbohydrate breakdown with the formation of lactic acid, the exposed surface was pigmented, there was greater resistance of the carious area to an explorer, and it could be fixed for histological study. This gross difference, that is, the firmer texture and pigmentation, cannot as yet be accounted for on the basis of the reported chemical findings, but seems to be associated with bacterial activity in the presence of glucose or its degradation products. In both instances whether the medium is maintained at a pH 4.0 by means of added buffer or by the addition of carbohydrate, the a-amino acid concentration as well as the ammonia (plus urea) nitrogen were similarly maintained. This suggests that the rate of utilization of a-amino acids is extremely slow in both experiments. The reason for this is as yet unknown. The possibility that added glucose was used as a nutriment thus resulting in a sparing of the a-amino acids is negated by the fact that a similar concentration of a-amino acids is maintained in the medium when a pH of 4.0 was effected by the addition of buffer but without addition of glucose. The similarity of the chemical changes occurring at a pH 4.0 suggests rather that this pH is not optimum for bacterial deami-

nation of a-amino acids. Since the a-amino nitrogen and ammonia (plus urea) nitrogen are similar in both experiments, the gross difference in the teeth can be attributed only to the presence of glucose or its degradation products. Whether the formation of lactic acid from added carbohydrate is the important chemical variation between these two experiments is as yet unknown. It is possible that there is a qualitative difference in the a-amino acids in these two experiments which might account for the gross difference in the appearance of these two teeth. Further studies on detailed chemical changes brought about by the presence of glucose are in progress.

SUMMARY

1. When human, intact teeth are incubated in a medium consisting primarily of known chemical substances including glucose and inoculated with a mixed oral flora, a pigmented, carious change develops on the exposed enamel surface and involves the underlying dentin. There is demineralization of the exposed enamel, however, surface continuity with the underlying destroyed dentin is maintained. This carious area is somewhat softer than that seen in human caries and probably resembles the acute type of caries. Microscopically, however, it resembles the changes occurring in the dentin and enamel in human in vivo caries. The chemical changes observed during incubation are the formation of lactic acid and calcium. Deamination of the amino acids in the medium appears to be inhibited.

2. When intact teeth are incubated in this inoculated medium without glucose there is no change observed on the exposed tooth surface. There is no calcium formed in the medium. Rapid deamination of the amino acids occurs and the medium becomes alkaline.

3. When intact teeth are incubated in this inoculated medium without glucose, but buffered at a pH 4.0, the exposed tooth surface becomes rapidly decalcified and except for the complete loss of enamel the change does not resemble natural caries. Calcium is formed and inhibition of deamination is again observed.

4. When intact teeth are incubated in this medium with glucose but buffered at a pH of 8.0 there is no change observed on the exposed tooth surface. There is no calcium formed and rapid deamination is observed.

5. In the above experiments glucose not only provides a substrate for acid formation thus producing a H-ion concentration suitable for decalcification of enamel, but also it appears to serve an entirely different role in that only when glucose is present in the medium are we able to produce the pigmented carious-like lesion involving the enamel and dentin.

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STUDIES ON THE EFFECT OF DL-GLYCERIC ALDEHYDE ON THE DENTAL CARIES ACTIVITY IN WHITE RATS AND COTTON RATS

JAMES H. SHAW

Harvard School of Dental Medicine, and Department of Nutrition, Harvard School of Public Health, Boston, Mass.

IN RECENT years, numerous research programs have been concerned with the search for compounds which would inhibit acid production by individual oral bacteria or by the collective flora in saliva. A considerable number of materials have been found which caused substantial reductions in the amount of acid formed by the oral flora in incubated saliva in comparison with the quantities of acid produced in control aliquots of saliva. One of the most interesting compounds to be tested in this manner was racemic glyceric aldehyde. Fosdick and Calandra[1] observed that the addition of increasing amounts of dl-glyceric aldehyde to saliva resulted in progressive decreases in acid formation during incubation. The test of acid formation used in these experiments was the ability to dissolve powdered human tooth enamel and was recorded as the amount of calcium brought into solution during the incubation period. In the tubes of saliva to which no glyceraldehyde was added, 24.0 mg. of calcium were found to be in solution in 100 ml. of saliva at the end of 4 hours; when as much as 200 or 250 mg. of racemic glyceric aldehyde were added to 100 ml. of saliva, only 3.5 mg. of calcium were present in the same volume after incubation. Intermediate additions to the incubation tubes resulted in intermediate amounts of dissolved calcium.

This striking reduction in the ability of the oral flora to produce acid in the presence of dl-glyceric aldehyde suggested the testing of this compound in experimental animals which were susceptible to the development of carious lesions. Two experiments were conducted in this laboratory with white rats and with cotton rats to determine if additions of racemic glyceric aldehyde to the diet had any effect on the initiation and rate of development of carious lesions in teeth which were known to be caries susceptible at the beginning of the experimental period.

EXPERIMENTAL PROCEDURE

Five litters of white rats of the Sprague-Dawley strain as modified in the Harvard Biological Laboratories were used as the subjects in the first experiment. These animals had been born to and suckled females which had been maintained throughout their reproductive lives on purified Ration 100 which has been described in detail previously.[2] Previous litters from the same females had indicated the attainment of a suitably high caries susceptibility in the offspring for this type of experiment. At least part of this observed susceptibility to tooth decay was a result of the feeding of the purified ration

Read at the 26th Annual Meeting of the International Association for Dental Research, Rochester N. Y., June, 1948 (*J. D. Res.* 27: 727, 1948).

Received for publication, May 16, 1949.

during early periods of tooth development as demonstrated by Sognnaes.[3] The 43 weanlings from these 5 litters were distributed into four groups. The 10 rats in the first group were maintained on purified Ration 100 for 14 weeks. The 11 rats in each of the second, third, and fourth groups received the same ration supplemented with 0.5, 1.0, or 2.0 per cent of crystalline dl-glyceric aldehyde, respectively. The albino rats in all groups were allowed access to food and water ad libitum.

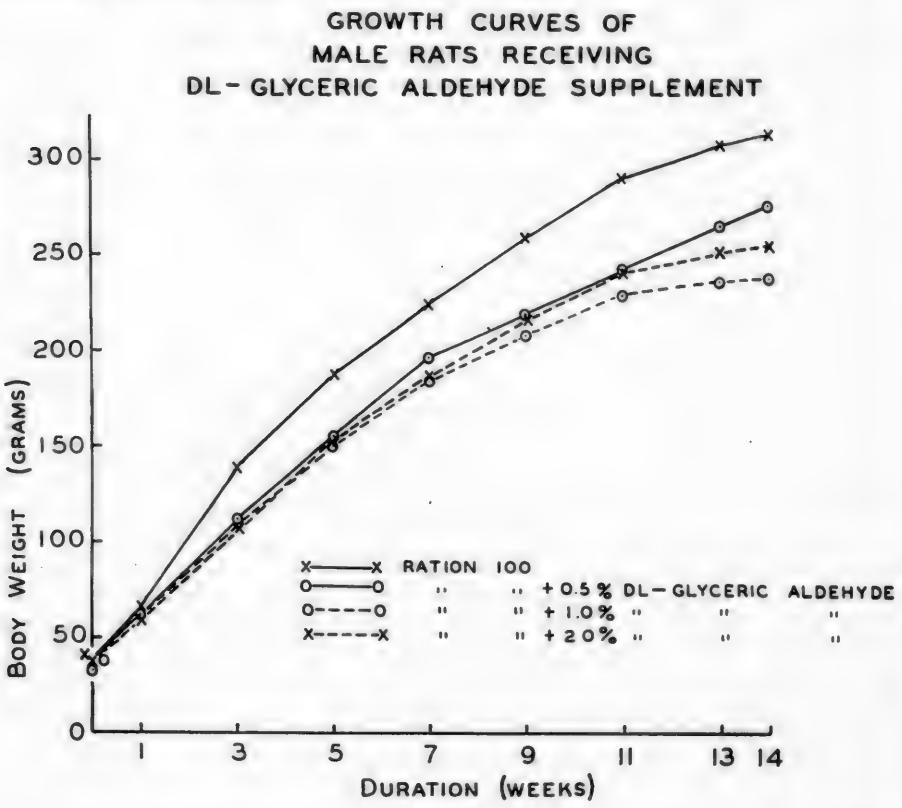

Fig. 1.

In the second experiment, 5 litters of weanling cotton rats were used as the test subjects. These animals had been born in our stock colony which is maintained on a ration of natural foodstuffs.[2] These 19 animals were distributed into four groups. The 5 cotton rats in the first group received Ration 100. The cotton rats in the second, third, and fourth groups received Ration 100 to which 0.5, 1.0, or 2.0 per cent of crystalline dl-glyceric aldehyde had been added, respectively. Since the available supply of racemic glyceric aldehyde was almost exhausted at the end of 9 weeks on experiment, the cotton rats in the third and fourth groups had to be transferred from the

diets supplemented with either 1.0 to 2.0 per cent levels to the diet containing 0.5 per cent glyceraldehyde for the remaining 5 weeks of the experiment. Food and water were provided to the cotton rats in individual cages ad libitum.

At the end of 14 weeks, the animals in both experiments were sacrificed by excess ether and the heads fixed in 95 per cent ethanol. After 48 hours, the heads were skinned and the molar teeth examined to determine the number and size of the carious lesions.

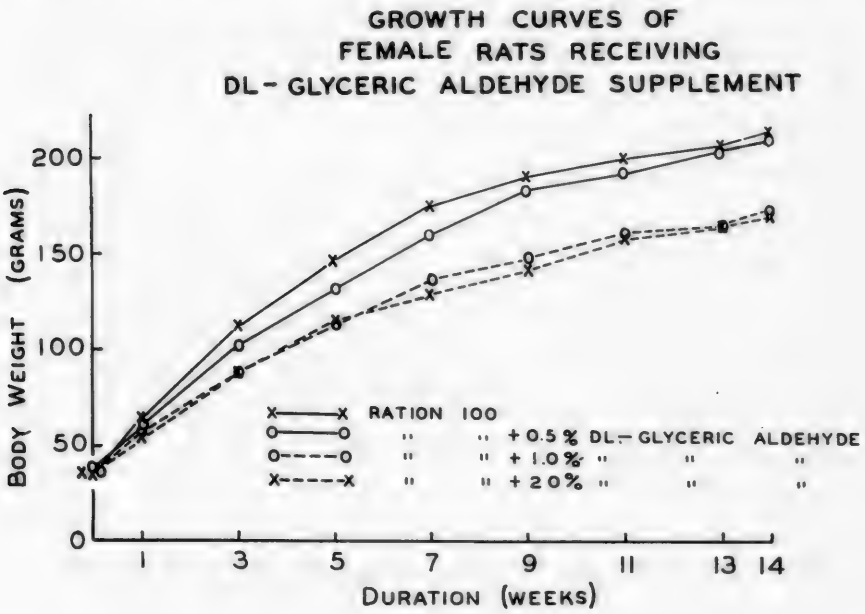

Fig. 2.

RESULTS

The average rates of growth for the male and female white rats in the first experiment are presented in Figs. 1 and 2. The male and female white rats receiving the supplements of dl-glyceric aldehyde grew at appreciably slower rates and attained lower final weights than their littermates in the control groups. The least effect on growth was noted in the female rats given the 0.5 per cent supplement which for some reason seemed to be somewhat less retarded in growth than the male rats receiving this amount. In view of the small number of males and females under consideration in this group, it is unlikely that any significance can be attached to the difference in response of the males and females to a 0.5 per cent glyceraldehyde supplement.

The average rates of growth of cotton rats were reduced to a lesser degree by glyceraldehyde supplements than those for the white rats. The 0.5 per cent supplement had no effect on the rate of growth in either male or females. The

1.0 and 2.0 per cent levels exerted a slight inhibitory effect during the first 6 weeks of the experiment. After that time, there was no demonstrable effect on growth with the final weights of all four groups being approximately the same as those of the control animals.

There was no other evidence of a toxic effect of the glyceraldehyde additions. Throughout the experimental periods, the animals receiving all levels of glyceric aldehyde supplementation were normal in appearance, hair coat, and activity.

The effect of additions of crystalline dl-glyceric aldehyde to Ration 100 on the initiation and rate of development in caries-susceptible white rats and cotton rats are presented in Tables I and II. In both species, the addition of 0.5, 1.0, or 2.0 per cent racemic glyceric aldehyde had no effect on the dental caries experience.

TABLE I

THE EFFECT OF ADDITIONS OF DL-GLYCERIC ALDEHYDE TO THE RATION ON THE DEVELOPMENT OF CARIOUS LESIONS IN THE WHITE RAT

SUPPLEMENT	NUMBER OF RATS	NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
		AVERAGE	S.E.M.*	C.R.†	AVERAGE	S. E. M.*	C.R.†
None	10	5.6	1.2	0.1	8.9 +	2.3 +	0.6
0.5% dl-glyceric aldehyde	11	5.5	0.8	1.1	10.7 +	1.9 +	0.8
1.0% dl-glyceric aldehyde	11	4.2	0.5	0.1	7.5 +	1.6 +	0.2
2.0% dl-glyceric aldehyde	11	4.9	0.8		8.3 +	1.6 +	

*Standard error of mean.
†Critical ratio.

TABLE II

THE EFFECT OF ADDITIONS OF DL-GLYCERIC ALDEHYDE TO THE RATION ON THE DEVELOPMENT OF CARIOUS LESIONS IN THE COTTON RAT

SUPPLEMENT	NUMBER OF RATS	NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
		AVERAGE	S.E.M.*	C.R.†	AVERAGE	S.E.M.*	C.R.†
None	5	25.0	1.5	1.4	66 +	9 +	1.1
0.5% dl-glyceric aldehyde	5	27.8	1.3	1.0	78 +	7 +	0.6
1.0% dl-glyceric aldehyde‡	4	27.2	1.4	1.9	73 +	6 +	1.4
2.0% dl-glyceric aldehyde‡	5	28.8	1.3		83 +	8 +	

*Standard error of mean.
†Critical ratio.
‡Reduced to 0.5 per cent dl-glyceric aldehyde for the last 5 weeks of the experimental period.

DISCUSSION

In dental research, there is a rapidly increasing necessity to conduct investigations which will evaluate the relationship, if any, between in vitro studies on the rate of acid production by the bacterial flora in saliva and in vivo experiments on the initiation and rate of development of carious lesions in caries-

susceptible laboratory animals. The relative economy, rapidity, and ease with which the in vitro tests can be conducted have made it readily possible to conduct literally hundreds of in vitro evaluations of the ability of many compounds to inhibit acid production by salivary bacteria. In in vivo animal experimentation, the cost is sufficiently high and the duration of the trials so long that much fewer tests can be made. If satisfactorily controlled, experimental evidence indicates that there is an absolute relationship between in vivo and in vitro experiments, the test-tube procedures could profitably be used for widespread investigation of possible inhibitory materials. Animal experimentation, then, could be used for final tests of necessary dosage, methods of application, etc. At the present time, insufficient data is available to permit a scientific evaluation of whether there is any satisfactory relationship between in vitro and animal experiments. A complete examination of the possible relationship would contribute considerable information to our present knowledge about the cause and methods of controlling dental caries.

Studies on glyceraldehyde were begun in this laboratory as one phase of a series of in vivo trials to evaluate materials of previously demonstrated inhibitory value with respect to acid production. The negative results obtained through the administration in the ration of crystalline glyceraldehyde to caries-susceptible white rats and cotton rats are in direct contrast to the extremely low amount of acid produced by the bacterial flora in saliva which is incubated in the presence of glyceraldehyde. One possible difference between the two procedures is the form in which the glyceraldehyde may have been present. Racemic glyceraldehyde is not known to have been produced in crystalline state in any other form than as the dimer. When the crystalline material is freshly dissolved in water, Reeves[4] has observed a molecular weight of 180.6 grams, that is, completely in dimeric form; at the end of a week in solution, measurements indicated a molecular weight of 90.3 grams, that is, a complete dissociation into the monomer. Intermediate data were not presented to indicate the rate at which dissociation from the dimer to the monomer occurred. For the purpose of evaluating the relation of glyceraldehyde to both test-tube and animal experiments, it would be of considerable value to know the exact rate of dissociation of the dimer to the monomer, especially during the first 24 hours in solution.

Presumably the crystalline glyceraldehyde mixed in the ration passed through the oral cavity of the experimental animals largely, possibly completely, as the dimer. In the test-tube experiments of Fosdick and Calandra,[1] some dissociation of the dimer to the monomer may have occurred during the four-hour incubation period, or the dl-glyceric aldehyde may have been added to the aliquots in a previously prepared solution where greater or complete dissociation to the monomer had occurred. At the present time, an experiment is underway in this laboratory to determine if dl-glyceric aldehyde given as the monomer to caries-susceptible animals will influence the initiation and rate of development of carious lesions.

SUMMARY

The addition of 0.5, 1.0, or 2.0 per cent of crystalline dl-glyceric aldehyde to a purified ration did not reduce the initiation or rate of development of carious lesions in caries-susceptible white rats or cotton rats.

This project was supported in part by a grant from the Sugar Research Foundation, Inc., New York. The 500 Gm. of dl-glyceric aldehyde used in these experiments were obtained for this purpose by Dr. R. C. Hockett, Scientific Director of the Sugar Research Foundation, Inc. We are indebted to Merck and Company, Inc., Rahway, N. J., for gifts of the B-complex vitamins used in this study.

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THE USE OF RADIOACTIVE PHOSPHORUS IN THE STUDY OF THE TEETH OF CARIES-RESISTANT AND CARIES-SUSCEPTIBLE STRAINS OF ALBINO RATS

CARL A. HOPPERT AND RAY L. SHIRLEY

Michigan State College of Agriculture and Applied Science
East Lansing, Mich.

HOPPERT, Webber, and Canniff[3, 4] demonstrated that dental caries could be developed in rats by feeding diets containing coarsely ground corn or rice. Subsequently, Hunt and Hoppert,[5, 6, 7] and Hunt, Hoppert and Erwin[8] made a genetic study in which two distinct strains of albino rats that vary markedly in their susceptibility to dental caries have been developed. The susceptible and resistant strains have been inbred through eighteen and fourteen generations, respectively. In the present investigation a comparison of the rates of deposition and removal of radioactive phosphorus in the teeth of these specialized strains and stock colony rats has been made. Also, the weight, ash, and phosphorus content of the teeth have been determined.

EXERIMENTAL AND RESULTS

General procedure.—In this investigation a comparison of the whole teeth of the resistant and susceptible strains and stock rats has been made with regard to (1) weight, (2) ash content, (3) phosphorus content expressed as per cent of dry weight of the teeth and of the ash, (4) rates of deposition and removal of radioactive phosphorus in the teeth after intraperitoneal injection of the isotope, (5) occurrence of labelled phosphorus in the teeth of offspring whose mothers were injected intraperitoneally with the isotope during pregnancy, and (6) adsorption of radioactive phosphorus in vitro by the teeth from aqueous solution. The weight, ash, and phosphorus values were obtained for the teeth of 58 susceptible, 53 resistant, and 42 stock colony rats in the age range of 6 to 60 days.

The rats were maintained on a cariogenic ration containing a more finely ground rice than originally used. The rice was so ground that 2 per cent was retained on a 20-mesh screen and 45 per cent on a 40-mesh screen. The corresponding values for the coarsely ground rice were 70 and 20 per cent, respectively. Since the rats of the susceptible strain would almost invariably have cavities forming by 60 days of age on the fine rice ration, this comparative study of the various strains was limited to rats 60 days of age or younger. Although the teeth do not erupt in young rats until about the fourteenth day, eight molars and the four incisors were removed from rats ranging from 6 to 14 days of age. In the case of older rats the four back molars were also included.

Read at a meeting of the Federation of American Societies for Experimental Biology, Detroit, Mich., April 18-22, 1949.

Taken from a thesis submitted by Ray L. Shirley to the Graduate Faculty of Michigan State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A joint contribution from the Kedzie Chemical Laboratory and the Department of Agricultural Chemistry published with the permission of the Director of the Agricultural Experiment Station as journal article No. 1043 (n.s.).

Received for publication, April 29, 1949.

Weight of teeth.—The molars and incisors were dried to constant weight in a hot air oven at 103° Centigrade. The weight of the molars increased steadily from 6 to 8 mg. at 6 days of age to approximately 60 mg. at 20 days of age after which time the rate of growth gradually decreased, whereas the incisors, having about the same initial weight as the molars, continued to increase at approximately 3 mg. per day throughout the period of observation, that is, 6 to 60 days. The weight of both the molars and incisors of the resistant strain tended to be slightly lighter in weight after 30 days of age than those of the susceptible strain and stock colony rats.

Per cent ash.—The teeth were removed, dried, weighed, and the per cent ash determined by heating the teeth in a muffle furnace at 600° Centigrade. Approximately 20 to 25 rats of each of the specialized strains and of the stock colony were sacrificed at 6 to 14 days of age. The ash of the molars increased from approximately 40 per cent at 6 days to 70 per cent at 14 days of age. The ash of the incisors increased from approximately 60 per cent at 6 days to 70 per cent at 14 days of age. No differences in ash values were observed among members of the same age in the three groups in the age range of 6 to 14 days. From 20 to 60 days of age, the concentration of ash in the teeth of each group was quite constant. However, as shown in Table I, the average ash values for resistant, susceptible, and stock colony groups were 78.8, 76.7 and 77.6 per cent, respectively.

TABLE I

ASH AND PHOSPHORUS VALUES OBTAINED IN THE AGE RANGE OF 20 TO 60 DAYS

STRAIN OF RATS	NO. OF RATS USED	PER CENT ASH		PER CENT PHOSPHORUS			
				DRY BASIS		IN ASH	
		MOLAR	INCISOR	MOLAR	INCISOR	MOLAR	INCISOR
Susceptible	37	76.7 ± 0.9	75.8 ± 1.1	15.0 ± 0.2	15.5 ± 0.3	19.6 ± 0.3	20.5 ± 0.4
Resistant	25	78.8 ± 0.9	76.4 ± 0.8	15.5 ± 0.4	15.5 ± 1.0	19.6 ± 0.5	20.3 ± 0.8
Stock	18	77.6 ± 1.0	76.4 ± 1.0	15.1 ± 0.4	15.6 ± 0.4	19.5 ± 0.4	20.3 ± 0.5

Per cent phosphorus.—The phosphorus in the teeth was determined by the phosphomolybdate method.[11] The ash residues were used for the analysis and the phosphorus values were calculated both as percentages of the dried teeth and of the ash. Phosphorus values were compared for teeth of the specialized strains and stock rats from 6 to 60 days of age. The per cent phosphorus in both the molars and incisors increased from 8 to 10 per cent at 6 days of age to approximately 15 per cent at 20 days of age at which time the values became constant for each group. As shown in Table I the molars of the resistant strain contained an average of 15.5 per cent phosphorus calculated on the dry weight basis as compared to 15.0 and 15.1 per cent for the susceptible and stock colony rats, respectively. However, on this basis essentially no difference was observed in the phosphorus content of the incisors.

When the phosphorus was calculated in terms of the per cent of the ash, no variation with age was obtained. As shown in Table I the three groups of rats gave similar values for per cent phosphorus in the ash in the case of both molars and incisors in the age range of 20 to 60 days. Comparable values were found in the case of a 167-day-old resistant female rat.

Deposition and removal of radioactive phosphorus in the teeth.—Radioactive phosphorus in the form of phosphoric acid in aqueous solution was obtained from Oak Ridge, Tennessee, after allocation by the Atomic Energy Commission. The radioactive solution was diluted to contain approximately 15 to 20 microcuries of radioactive isotope per milliliter. One milliliter of this solution had an activity of approximately 1,600,000 counts per minute. In this investigation the radioactive phosphorus was evaluated with a Bale-Haven immersion-type Geiger-Mueller tube in conjunction with a modified Neher-Harper quenching circuit and Tracerlab, Inc., Autoscaler counter.

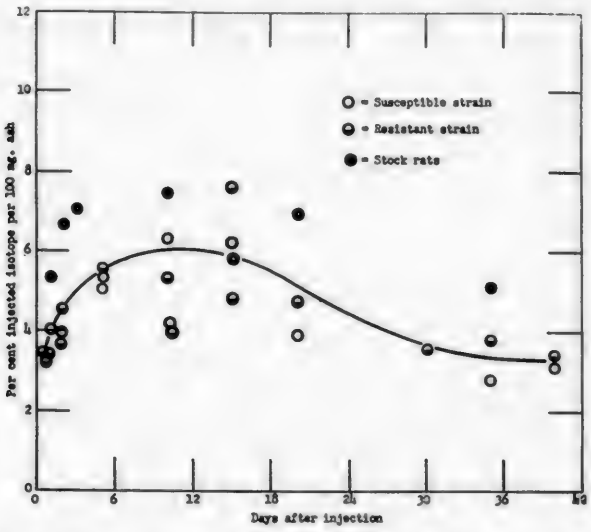

Fig. 1.—Rate of deposition and removal of phosphorus-32 in molars of rats of the various strains that were administered isotope at 20 days of age.

One milliliter of the radioactive isotope solution was injected into the peritoneal cavity of resistant, susceptible, and stock rats at two different ages, namely, at 20 and 35 days of age. At intervals of a few hours to 40 days after administration of the isotope the animals were sacrificed, the teeth removed, cleaned, ashed at 600° C., and the radioactivity of an acid solution of the ash determined. The results were calculated as per cent of administered dose recovered in the teeth per 100 mg. of ash at the time of sacrifice. Figs. 1 and 2 show graphically the results obtained for molars and incisors, respectively, of the animals that were injected with the isotope at 20 days of age. Figs. 3 and 4 show graphically the data obtained on the molars and incisors, respectively, of the corresponding rats that were injected with the isotope at 35 days of age.

As shown in Fig. 1, the molars of the rats that were administered the isotope at 20 days of age contained approximately 4 to 7 per cent within 24 to 48 hours. The per cent deposition increased slightly up to 15 days after which time the concentration of the isotope began to decrease. Fig. 2 shows that the corresponding incisors had a deposition of approximately 5 to 9 per cent of the injected isotope within 24 to 48 hours, and that the value tended to increase

slightly up to 5 to 10 days after which time the concentration decreased gradually to 3 to 4 per cent after 30 to 40 days. Two resistant strain rats of this group were found to have approximately 0.02 per cent of the isotope present in both the molars and incisors 97 days after administration.

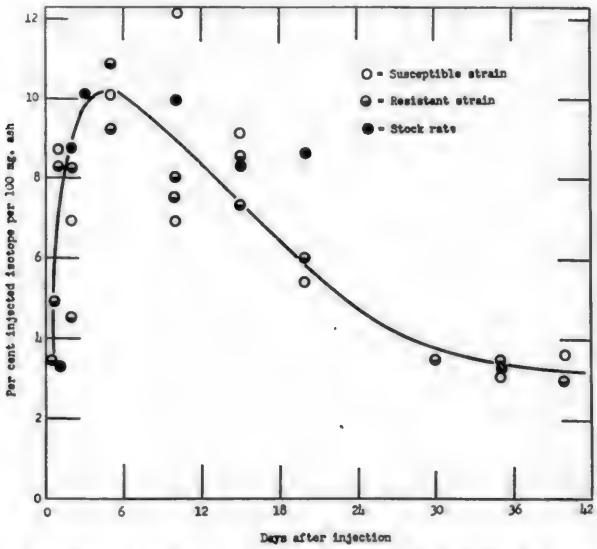

Fig. 2.—Rate of deposition and removal of phosphorus-32 in incisors of rats of various strains that were administered isotope at 20 days of age.

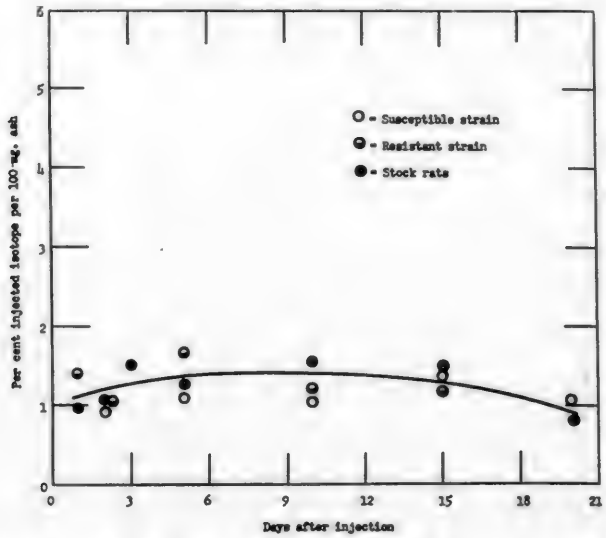

Fig. 3.—Rate of deposition and removal of phosphorus-32 in molars of rats of various strains that were administered isotope at 35 days of age.

Fig. 3 shows that the specialized strains and stock rats that received the isotope at 35 days of age had approximately 1 per cent of the dose deposited

in the molars at 24 to 48 hours following the administration. The per cent deposition of the isotope was constant at approximately 1 to 1.5 per cent from 5 to 15 days after administration after which time the concentration decreased. As shown in Fig. 4 the incisors of the resistant strain contained slightly more of the isotope than those of the stock and susceptible strains. The incisors in general contained 2 to 3 times more labelled phosphorus than the corresponding molars. The rats that were administered the isotope at 35 days of age deposited only one-third to one-half as much in their molars and incisors as the rats that were injected at 20 days of age.

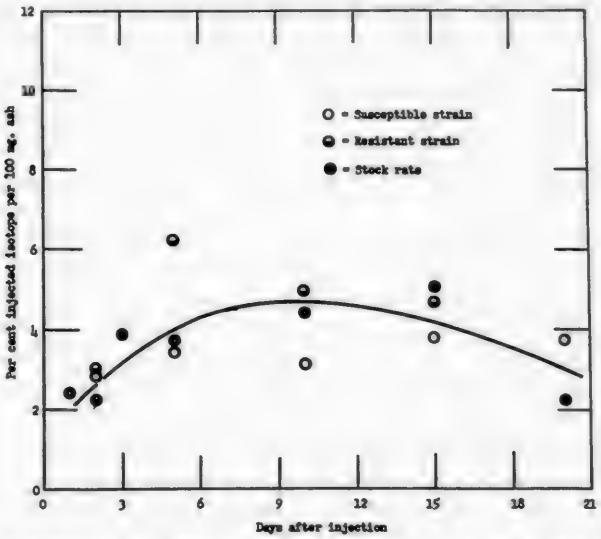

Fig. 4.—Rate of deposition and removal of phosphorus-32 in incisors of rats of various strains that were administered isotope at 35 days of age.

Radioactive phosphorus found in teeth of offspring of mothers that had been administered the isotope during pregnancy.—In this experiment pregnant females of the resistant and susceptible strains and stock rats were injected intraperitoneally during pregnancy with 5 ml. of an isotope solution which contained approximately 90 to 100 microcuries of radioactive phosphorus. The offspring were sacrificed at 6, 9, 14, and 20 days of age, and the activity in the whole molars and incisors determined. The data obtained indicated that the offspring were normal in regard to weight, ash, and total phosphorus in the teeth at time of sacrifice. An appreciable amount of radioactivity was found in the teeth of all offspring. However, the earlier in the gestation period the injection was made the lower the percentage of isotope found in the teeth of the offspring.

A direct comparison of the susceptible and resistant strains was made possible by the birth of a litter of each strain two days after the pregnant females were administered the isotope. The data obtained with the molars and incisors of these two litters are shown graphically in Figs. 5 and 6, respectively. Fig 5 shows that the per cent of administered isotope recovered in the molars per 100 mg. of ash decreased in both strains from about 5 per cent at 6 days of age to

approximately 2.1 per cent at 20 days of age. As shown in Fig. 6 the labelled phosphorus decreased in the incisors of both strains from 6 to 7 per cent at 6 days of age to approximately 2.4 per cent at 20 days of age.

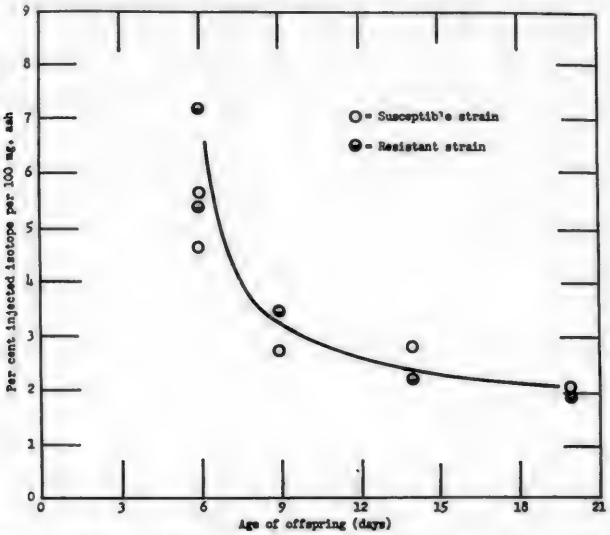

Fig. 5.—Phosphorus-32 found in molars of young rats of various strains whose mothers were administered isotope during pregnancy.

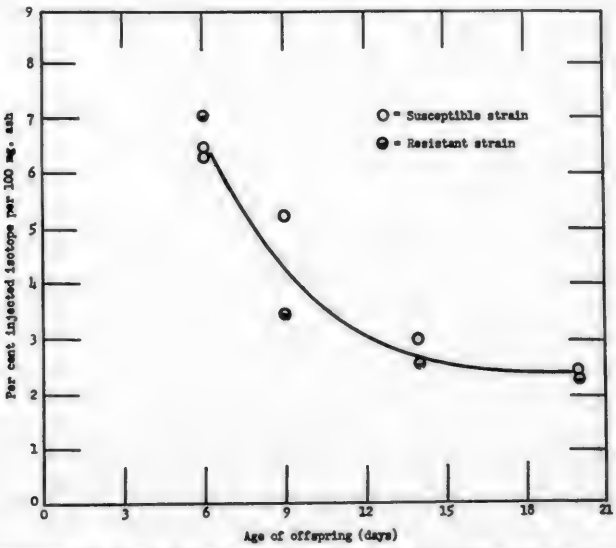

Fig. 6.—Phosphorus-32 found in incisors of young rats of various strains whose mothers were administered isotope during prenancy.

In vitro adsorption of radioactive phosphorus.—In this experiment the teeth of the specialized strains and stock rats were tested for in vitro adsorption of the isotope by a procedure similar to that used by Hodge, Van Huysen, Bonner,

and Van Voorhis.[2] The teeth were extracted with an ethylene glycol solution of 3 per cent potassium hydroxide to remove the organic matter. After filtering off the extracting solution the teeth were ground to medium fineness and agitated for 8 hours in 25 ml. of neutral aqueous solution of radioactive sodium hydrogen phosphate at 40° Centigrade. The radioactive solution had an activity of approximately 2,000 counts per minute per 5 ml. as determined with the immersion-type Geiger-Mueller tube used in this investigation. After exposure the ground dental material was washed, dissolved in dilute hydrochloric acid, and analyzed for phosphorus-32.

The data obtained are presented in Table II. The teeth of the 60-day-old rats adsorbed appreciably less phosphorus than those of the younger rats. Both the molars and incisors adsorbed radioactive phosphorus equivalent to 20 to 30 counts per minute per mg. of ash, whereas the teeth of the 20-, 30-, and 40-day-old rats adsorbed sufficient isotope to give in the range of 50 to 60 counts per minute in the case of the molars and 70 to 80 counts per minute in the case of the incisors.

TABLE II

IN VITRO ADSORPTION OF RADIOACTIVE PHOSPHORUS

STRAIN	AGE (DAYS)	WT. OF TEETH (MG.)		PER CENT ASH*		ADSORPTION OF P-32 (C/M/MG. ASH)†	
		MOLAR	INCISOR	MOLAR	INCISOR	MOLAR	INCISOR
Susceptible	20	61.8	43.6	85.1	78.4	46.8	78.6
Stock	20	58.1	40.5	83.8	79.3	54.9	73.9
Stock	20	53.8	38.1	82.9	82.2	56.3	70.3
Susceptible	30	81.6	60.0	85.2	86.5	54.6	83.0
Resistant	30	73.4	59.4	86.2	87.2	57.9	77.8
Resistant	30	72.1	61.6	86.7	86.2	63.7	81.7
Susceptible	40	114.4	—	88.3	—	57.2	—
Resistant	40	93.2	—	84.7	—	54.8	—
Susceptible	60	120.3	159.8	82.6	84.7	29.3	20.3
Resistant	60	105.8	119.7	84.4	86.5	27.2	24.2

*Value obtained by extraction of organic matter with 3 per cent potassium hydroxide in ethylene glycol.

†Counts per minute per mg. of ash.

DISCUSSION

Very little chemical work has been done on the teeth of the caries-resistant and caries-susceptible strains of albino rats previous to this investigation. Whole teeth only were used in this study in order to determine if gross chemical differences existed between the strains. Subsequent chemical studies of the teeth will be concerned with comparison of the enamel and dentin.

A few ash determinations made by extracting the organic matter from the teeth with 3 per cent potassium hydroxide in ethylene glycol gave 5 to 8 per cent higher values than those obtained by ashing corresponding teeth at 600° Centigrade. Bowes and Murray[1] round that ashing at high temperatures expelled all the combined carbon dioxide in the dentin and about 75 per cent of that in the enamel. They reported that dry dentin and dry enamel contained 3.18 and 1.95 per cent of combined carbon dioxide, respectively. These investigators also reported that extraction methods remove approximately 0.4 per cent of the phosphorus as organically combined phosphorus. Further work is necessary to deter-

mine if some particular fraction of the molars of the specialized strains is responsible for the small differences in ash and phosphorus encountered in this investigation. The data obtained in this study indicated that the phosphorus content of the ash in the whole teeth is independent of age, sex, and heredity.

The variation in the capacity of members of the same strain to deposit and remove radioactive phosphorus from the teeth indicates that small differences between the strains cannot be determined easily. The apparent biological variation encountered in this study is of the same order as that obtained by Manly and Bale[10] when they gave radioactive phosphorus by stomach tube to albino rats and determined the rate of deposition and removal of the isotope in the teeth.

The results of the experiment in which the two specialized strains were compared in regard to the occurrence of labelled phosphorus in the teeth of the offspring of mothers that had been administered the isotope during pregnancy indicates that the early phosphorus metabolism of the teeth of the two strains is very similar. Sognnaes and Volker[12] demonstrated that equal weights of the enamel and dentin of unerupted teeth of a monkey adsorbed the same amount of radioactive phosphorus. Since the teeth were removed from the young rats in the present experiment in the pre-erupted stage or soon afterwards it might be assumed that the enamel and dentin of the two strains are equivalent in their capacity to assimilate phosphorus at least in the early period of their development.

Hodge, Van Huysen, Bonner, and Van Voorhis[2] found that albino rat teeth adsorbed radioactive phosphorus in vitro from an aqueous solution in accordance with the Freundlich adsorption isotherm. Subsequently, Johansson, Falkenheim, and Hodge[9] showed that when enamel and dentin were exposed to radioactive phosphorus in vitro fairly rapid adsorption of the isotope occurred in each tissue over a period of at least 8 hours. For this reason, the teeth were exposed to the labelled phosphorus in aqueous solution for 8 hours in the in vitro adsorption tests made in the present investigation.

CONCLUSIONS

The data obtained in this investigation of the whole teeth of two strains of albino rats that are markedly different in their susceptibility to dental caries indicate that they vary slightly in the following respects: (1) Weight of teeth. (2) Per cent ash in the molars. (3) Per cent phosphorus in the molars.

They were found to be essentially similar in the following respects: (1) Per cent ash in the incisors. (2) Per cent phosphorus in the incisors on the dry weight basis, and per cent phosphorus in the ash of the molars and incisors. (3) Rate of deposition and removal of radioactive phosphorus in the teeth when the isotope was administered intraperitoneally. (4) Occurrence of radioactive phosphorus in the teeth of offspring of females that were injected intraperitoneally with the isotope during pregnancy. (5) In vitro adsorption of the isotope by the teeth from aqueous solution.

The writers wish to thank Dr. H. R. Hunt, of the Department of Zoology, for kindly supplying the resistant strain rats used in this investigation, and Dr. E. J. Miller and Dr. E. J. Benne, of the Department of Agricultural Chemistry, for helpful suggestions and aid in making the tracer assay equipment available.

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THE AMMONIA CONTENT OF THE MOUTH

W. E. LUDWICK*† AND L. S. FOSDICK

The Clinics and Laboratories of Northwestern University Dental School, Chicago, Ill., and the Dental Department, U. S. Naval Training Center, Great Lakes, Ill.

IT HAS been quite adequately shown that an ammonium-ion concentration of 50 mg./100 c.c. will effectively inhibit the growth of bacillus acidophilus.[1, 2] Furthermore, it has been shown that under the proper conditions of use the introduction of ammonium ion into the oral cavity will usually cause a decrease in the acidophilus content of the saliva. On the basis of the above evidence several dentifrices that are a source or potential source of the ammonium ion have been formulated.

It has been suggested that the controlling factor in natural immunity to dental caries is the ammonium content of the mouth. The first proposal of this nature by Grove and Grove[3, 4] explained the action on the basis that ammonia would dissolve the so-called mucin plaques. One of the main criticisms of this theory is that the saliva never contains a sufficient amount of ammonia to inhibit the growth of lactobacillus acidophilus or to dissolve the mucin plaque. The ammonium concentration of the saliva has been quite thoroughly investigated[5] and it is found to vary between 0 and 20 mg. per 100 c.c., and in no case has it been reported as high as the necessary 50 mg./100 c.c. In those studies in which the ammonium concentration of saliva from immune cases has been compared with the ammonium concentration of caries-active cases, there has been little correlation. In view of this discrepancy it was suggested by Kesel[6] that the ammonium ion was not necessarily in the saliva of immune individuals in sufficient concentrations to inhibit the growth of lactobacillus acidophilus, but could be concentrated in the dental plaque. This is a logical hypothesis, as a variable concentration of the hydrogen ion in the various portions of the mouth has been shown to exist.[7, 8] Thus high acid potentials may exist in the caries-active areas of the mouth while the rest of the mouth and the oral secretions are essentially neutral. It may be argued that insofar as high acid potentials may exist in isolated portions of a normally neutral mouth, so might high ammonium ion concentration coexist with a normally ammonium-free saliva, or saliva with low concentrations of ammonium. In view of the above it was decided that if the ammonium ion concentration on the tooth could be determined, and that of caries-immune cases compared with that of caries-active cases, the accuracy of the theory could be tested.

The determination of the ammonium-ion concentration in isolated plaque material would be exceedingly difficult, if not impossible, with the present

This work was partially supported by grants from the Colgate Palmolive Peet Company.
Received for publication, April 18, 1949.

*Commander, DC, USN.

†The opinions and assertions contained in this article are the private opinions and assertions of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. Article 113, U.S. Navy Regulations.

methods of analysis. One of the recognized methods of ammonia determination involves the absorption of the ammonium ion onto an abrasive-like material, permutit. The permutit, a synthetic aluminum silicate, when washed with acid, will rapidly absorb ammonium ions, even in the presence of high salt concentrations. The absorbed ammonium ions can then be readily displaced with a strong alkali, after which the free ammonium ion can be determined by many methods. It was thought that if the teeth were brushed using permutit as the dentifrice, the "dental plaques" would be removed as far as is possible by brushing the teeth, and that the ammonia content of the plaque material would be quantitatively transferred to the permutit. The ammonium content of the brushings would then be a function of the ammonium content of the plaque. Furthermore, if the ammonium concentration of the plaque were constant, then by brushing the teeth with permutit and subsequently removing all of the plaque by mechanical means and analyzing for ammonia, a method of determining the cleansing effect of brushing the teeth would be available.

EXPERIMENTAL

Two procedures were used in the determination of the ammonium content of the material from the tooth surfaces. The first procedure was a simple determination of the ammonium content of tooth brushings. The second was the determination of the ammonium content of tooth brushings plus that which could be removed by the use of dental instruments. For the first experiment 1000 cases were selected at random from young naval recruits and each was given bite-wing radiographs, a complete dental examination, and salivary acidogenic bacteria count. In addition to this each individual was instructed to brush his teeth for a period of one minute, immediately upon arising, using a measured amount of permutit as the dentifrice. All of the permutit was immediately expectorated into a flask, after which the mouth and the brush were rinsed and the rinsings placed within the same flask. The mucous material and debris were removed by thrice washing the permutit, after which the permutit was collected and placed in a volumetric flask. The ammonia was liberated by means of 10 per cent NaOH and the ammonium-ion concentration was determined by means of the Nessler reagent. The color intensity was measured by means of a Coleman spectrophotometer.

In the second experiment fifty selected individuals were given a similar treatment with the exception that in each case the teeth were given a complete prophylaxis and all of the scalings from the tooth surface were rinsed into the flask containing the washed permutit. For this purpose five daily consecutive tests were made on each individual for the ammonium content of tooth brushings. The teeth were brushed immediately upon arising in the morning before the individual had received any food, and before any smoking or anything other than water had been taken into the mouth. The daily variations were noted and it was found that the daily ammonium content of the brushings was surprisingly constant. Immediately after the fifth brushings a complete prophylaxis was given and the tooth scrapings were added to

the permutit from the brushings and the total ammonium content was determined. These data would yield the total amount of ammonium on the tooth surface plus any accumulation of ammonia from the saliva secreted during the brushing interval. These data would also yield information concerning the relative amount of debris that could be removed by brushing the teeth.

RESULTS

Table I shows the average ammonium concentration of the brushings from the caries-active and caries-immune individuals. Caries activity was determined by the aciduric bacteria counts on the saliva of the individuals and by clinical examination. The aciduric bacteria counts from 0-10,000 represented the relatively immune individuals and counts above 10,000 were considered as caries active. The clinical estimations of caries activity were zero, plus one, plus two, plus three, and plus four, depending upon the number of open cavities, fillings, and the general appearance of the mouth. For the purpose of statistical analysis, clinically immunes were regarded as zero and plus one, and the very actives were plus two, plus three, and plus four; by the use of the bacteria count there were 388 relatively immune individuals and 612 relatively caries-active individuals. By means of the clinical examinations there were 300 cases of clinically inactive caries and 700 cases of marked caries activity. As can be seen from the above tables, those who were immune as determined by the lactobacillus acidophilus counts contained .33 mg. of ammonia, while the caries-active cases contained .32 milligrams. Those who were caries active as judged by clinical examinations had an ammonium content of .32 mg. while the caries-inactive cases contained a content of .32 milligram. On the basis of this investigation it would seem that there are no significant differences between the ammonium content of the tooth brushings of individuals with active caries and those with inactive caries.

TABLE I

MEAN AMMONIA CONTENT OF TOOTH BRUSHINGS

TYPE CASE	NUMBER OF CASE	MEAN AMMONIA
Clinically immune	300	.32
Clinically susceptible	700	.32
Bacteriologically immune	388	.33
Bacteriologically susceptible	612	.32

By the use of the second procedure 25 caries-immune cases were compared with 25 caries-susceptible cases. It was found that the daily variations in ammonium content of the tooth brushings were surprisingly constant; the daily variation being about 10 per cent. Table II indicates the results of this test. The ammonium content of the brushings varied from .33 mg. for the susceptible cases to .37 mg. for the immune cases. The total ammonium content including the brushings and the scalings varied from .41 mg. to .44 mg. Thus, about 20 per cent of the ammonium is concentrated in those areas not accessible to the brush. There was no difference between the total ammonium content of caries-active and caries-immune cases.

TABLE II

TOTAL AMMONIA CONTENT OF MOUTH

TYPE CASE	NUMBER OF CASES	MEAN NH_3 BRUSHING	NH_3 BRUSHING AND SCALING	% NH_3 REMOVED
Clinically immune	25	.37 mg. ± .046 mg.	.41 mg.	90
Clinically susceptible	25	.33 mg. ± .042 mg.	.44 mg.	75
Bacteriologically immune	25	.37 mg. ± .044 mg.	.43 mg.	86
Bacteriologically susceptible	25	.36 mg. ± .048 mg.	.42 mg.	86

DISCUSSION

On the basis of the experimental results, it would seem that the ammonium concentrations on the surface of the teeth do not differ between caries-active and caries-immune individuals. This was evident when the total ammonia of the mouth was determined and also when only that which could be removed by means of a tooth brush was estimated. Although the results seem to furnish evidence that the ammonia theory of caries immunity is incorrect, it by no means provides conclusive evidence, as there are several factors that would tend to invalidate the conclusions. There is little question that under the conditions of the experiment all of the available ammonia was absorbed by the permutit. However, it is certain that all of the ammonia did not originate on the surface of the teeth. The saliva that is secreted during the brushing period would in some cases be appreciable. Preliminary investigation along this line indicated that this was not an appreciable factor in the results. It is quite certain that desquamated epithelial cells and perhaps other debris on the cheeks and tongue may also be a factor. No evidence was obtained concerning this factor; however, if it was a factor it was quite constant as shown by the results of daily brushings. When we consider that almost .5 mg. was removed from the mouth, it is quite obvious that this ammonium concentration in the oral cavity is much higher than is ordinarily found in saliva. Furthermore, 20 per cent of the entire oral ammonia is found in those places inaccessible to the brush.

It is quite possible that relatively large concentrations of ammonia may be present in extremely small areas and hence be masked by a low ammonium content of a large area. Thus, although there appears to be no difference in the total amount of ammonia in immune mouths as compared with susceptible mouths, it is not conclusive evidence that ammonium plays no part in immunity to caries. Nevertheless, it is an indication that the total mouth ammonia is not associated with caries activity.

It is of much interest to note that 80 per cent of the total ammonia can be removed by brushing the teeth. There is no data on the relative area of the proximal surfaces. It would seem that an 80 per cent removal of the debris from the teeth by brushing would be more than is usually expected.

SUMMARY

The total mouth ammonia and that which can be removed by brushing have been determined. This study revealed no correlation between the total ammonia content of the mouth and caries activity.

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THE OCCURRENCE OF BETA HEMOLYTIC STREPTOCOCCI ON THE GINGIVA OF NORMAL YOUNG ADULTS

MAURY MASSLER, D.D.S., M.S., AND JOHN B. MACDONALD, D.D.S., M.S.

University of Illinois, Research and Educational Hospital, Chicago, Ill.

IT HAS become increasingly evident during the past twenty-five years that the factors operating in the spread of air-borne infection are numerous and complex. The importance of localization of pathogenic organisms on various loci of the upper respiratory tract has been emphasized in work reviewed by Duguid (1946), namely, Bloomfield and Felty (1924), Colebrook (1933), Paine (1935), and Hare (1940). All of these investigations showed that only a small percentage of the throat carriers of hemolytic streptococci expelled these organisms during speaking and coughing. The importance of localization was further emphasized by Jennison (1942) who argued that most of the expelled droplets must originate from the front of the mouth rather than from the throat. Duguid (1945) showed that when *Salmonella marcescens* was used as an indicator, most expelled droplets actually did originate from the front of the mouth (lips, teeth, and tip of tongue). Hamburger, Green, and Hamburger (1945) showed that in 400 hospitalized patients with hemolytic streptococcus infection of the upper respiratory tract, eighty times as many hemolytic streptococci were expelled by patients with strongly positive nasal cultures as by patients with positive throat and negative nasal cultures. All the above evidence led Duguid (1946) to the conclusion that "The danger of air-borne infection should be assessed not in terms of the occurrence of *throat carriage* of pathogenic organisms but in terms of the occurrence of *oral carriage* and *nasal carriage.*" He observed also that "Infection of the nasal cavity and of the anterior mouth does not occur in regular association with infection of the throat."

In view of these reports it seemed worth while to examine a group of normal individuals to determine the relative occurrence of beta hemolytic streptococci on the labial gingiva of the lower anterior teeth and on the surface of the amygdaloid fossa.

Occurrence of Beta Hemolytic Streptococci in the Throat.—An examination of the literature covering investigations of the occurrence of beta hemolytic streptococci in and on the throat discloses a wide range of figures. Occurrence rates as reported vary from 0.3 per cent (Paul and Freese, 1933) to 63 per cent (Towers, Wei, and Yu, 1940). The former study was on about 500 residents of an Arctic Island and the latter was on over 300 nurses in Shanghai. Wu (1941) presented an excellent compilation of the results of sixteen such studies. Other noteworthy studies of normal populations not included in the review by Wu have been reported by Shibley, Hanger, and Dochez (1926), Rhoads and Afremow (1943) and by Van Ravensway (1944).

This investigation was supported in part by a grant from The Division of Research Grants and Fellowships of the National Insititute of Health of the United States Public Health Service.

Received for publication, March 30, 1949.

The reasons for the wide variation in occurrence rates are several. The groups cover widely divergent geographic and consequently climatic localities including arctic, temperate, and tropical zones. They were studied at different times of the year and seasonal variation in carrier rates have been frequently reported, for example, Wu (1941). Although all groups were apparently healthy they varied in years from childhood (Bailey, 1939) to old age (Davis and Guzdar, 1943). Lack of standardization of method undoubtedly contributed in some measure to the varied results as pointed out by Topley and Wilson (1946).

Occurrence of Beta Hemolytic Streptococci Within the Oral Cavity.—Studies of the occurrence of beta hemolytic streptococci within the oral cavity are notably lacking. Blackburn, Boston, Gilmore, Lovell, and Smith (1930) in a study of a normal Manchester population reported the carrier rates for these organisms as obtained by nasopharyngeal swabs and by mouthwashings to be practically identical, about 11 per cent.

Hamburger, Puck, Hamburger, and Johnson (1944) examined the saliva of 156 patients, 80 of whom had scarlet fever and 76 of whom had streptococcal pharyngitis or tonsillitis. He found that the saliva in these cases might or might not contain demonstrable hemolytic streptococci. When hemolytic streptococci were found in the saliva, the numbers varied widely from case to case. In about one-half of the patients, the number of hemolytic streptococci diminished gradually from day to day. In 25 per cent there was no change over several weeks. In this group, one-half showed the presence of hemolytic streptococci and one-half did not. Tonsillectomized patients carried fewer hemolytic streptococci in the saliva and for a shorter time than patients with tonsils.

Duguid (1946) investigated the hemolytic streptococcus content of mouth-spray droplets in 87 patients with these organisms present in the throat. He also examined throat swabs and swabbings from the inner surfaces of the lips, inner and outer surfaces of the teeth, and tip of the tongue. Hemolytic streptococci were found in the anterior mouth secretions of 13 of the 87 patients (15 per cent). Infected droplets were expelled by 39 of the patients in one or more of a series of six coughs.

Methods of Culturing.—There is at present no uniformity of method for investigations of the occurrence of hemolytic streptococci. Serologic technics are too burdensome for large scale surveys and investigators have generally relied on the appearance of streptococcus colonies on streaked blood agar plates. Brown (1919) strongly recommended the use of poured blood agar plates. Pike and Leonard (1945) compared the streaked plate with streak-pour plates. The latter combines the advantages of the poured plate with a simplicity approaching that of the streaked plate. From 159 cultures of throat swabs by both methods, 39 per cent showed growth of hemolytic streptococci on streaked plates and 41 per cent on streak-pour plates. This study suggests that poured plates have no great advantage over streaked plates for the survey type of investigation in which presence or absence of hemolytic streptococci are more important than the absolute numbers of such organisms.

The present study was aimed primarily at determining the occurrence of beta hemolytic streptococci on the gingiva of normal young adults as compared with the occurrence of these organisms on the throats of the same group. Since the investigation was a comparative one and not directed at determining absolute figures and since it was of interest to know whether useful information could be gained by routine swabbing of the gingiva as well as the throat in diagnostic laboratories, it was decided to use a method of isolation common to diagnostic laboratories.

METHOD AND MATERIALS

The medium used was 10 per cent citrated human blood in extract agar containing 0.2 per cent dextrose. Since human blood may contain antihemolysins (e.g., Pike and Leonard, 1945) each batch of plates was tested before use by streaking with a beta hemolytic streptococcus stock culture (Group C). Where hemolysis was inhibited the blood was not used. The subjects investigated were 500 medical and dental students (predominantly males) at the University of Illinois. The period of the investigation was from January to April of 1948.

A cotton swab on a wooden applicator stick was stroked over the region of one tonsillar fossa of each student. The swab was then rotated on a small area of the periphery of a blood agar plate. Another swab was then stroked over the labial gingiva of the lower anterior teeth of the same individual and the material transferred to the opposite side of the same plate previously divided into halves with a wax pencil. Within two hours of the time of inoculation, the plates were streaked by means of a wire loop in a continuous, close spiral. They were incubated for 24 hours at 37.5° C. and then examined in good artificial light.

Colonies of streptococci surrounded by a zone of complete sharply defined hemolysis were classified as beta hemolytic streptococci. Colonies surrounded by zones of incomplete or ill-defined hemolysis were classed as "partially hemolytic colonies." Some of these latter on examination of stained smears proved to be other than streptococci.

FINDINGS

Occurrence of Beta Hemolytic Streptococci.—Beta hemolytic streptococci were observed in cultures from the throats of 58 cases and on cultures from the gingiva of 30 cases. The percentage of occurrence for this organism on the throat was therefore 11.6 per cent and on gingiva 6 per cent (in 500 cases). The probability of such a difference occurring as the result of chance selection in a series of 500 cases is less than 1 in 100. The difference between throat and gingiva in this respect is shown also by the distribution of the positive cultures. The organism was observed on cultures from 77 individuals—a percentage occurrence of 15.4 per cent in 500 cases. It was found on the throat alone in 47 cases, on the gingiva alone in 19 cases, and on both throat and gingiva in only 11 individuals (Table I). In this series, therefore, the occurrence of beta hemolytic streptococci was not the same on the gingiva as on the throat. The organisms, when present, were usually on the throat or on the gingiva but seldom on both sites at the same time.

TABLE I

OCCURRENCE AND DISTRIBUTION OF BETA HEMOLYTIC STREPTOCOCCI ON THE THROAT AND GINGIVA OF 500 NORMAL YOUNG ADULTS

Total number of persons examined	500		
Number of positive cultures	88		
From throat	58	(11.6%)	
From gingiva	30	(6.0%)	
Number of persons with positive cultures	77	(15.4%)	
On throat alone		47	(61.1%)
On gingiva alone		19	(24.6%)
On both throat and gingiva		11	(14.3%)

DISCUSSION

Duguid's (1946) conclusion that the occurrence of beta hemolytic streptococci in the anterior secretions of the mouth does not parallel the occurrence in the throat is supported by the findings reported here. It has been generally assumed, however, that the source of beta hemolytic streptococci in the saliva or anterior oral secretions has been the throat. The present evidence casts an element of doubt on this matter since in about one quarter of the positive cases cultures from the gingivae disclosed the organism where the throat was negative. This does not necessarily mean that these streptococci can survive indefinitely on the gingivae independently of the throat. The method could only give results too low rather than too high because of the swab missing localized areas of colonization. Furthermore, it is by no means impossible that these individuals had recently been throat carriers and had at the time of investigation become negative as far as the throat was concerned. Hamburger, Puck, Hamburger, and Johnson (1944) in fact observed six "positive salivas" amongst 39 negative throat cultures where the throats had previously harbored the organisms. If alternatively the streptococci do not multiply in the normal mouth and oral "contamination" from the throat is intermittent, both oral presence and expulsion might depend on repeated coughing, etc. The question is complex and serial studies of both types of carrier are needed to supply some of the answers.

Regardless of the answers, it would seem wise to examine both throat and gingiva where beta hemolytic streptococci are being sought amongst a group of potentially dangerous carriers (e.g., obstetrical ward personnel) or where their presence may be significant in disease processes (e.g., rheumatic fever or acute glomerulonephritis).

SUMMARY AND CONCLUSIONS

A series of 500 students were subjected to bacteriological investigation of the tonsillar fossa and gingiva of the lower anterior teeth for the presence of beta hemolytic streptococci. It was found that beta hemolytic streptococci were disclosed on the throat in 58 cases (11.6 per cent) and on the gingiva in 30 cases (6 per cent). Of the 77 cases in which beta hemolytic streptococci were found, the organisms occurred on throat alone 47 times (61 per cent), on the gingiva alone 19 times (24 per cent), and on both throat and gingiva only 11 times (14 per cent).

It is concluded that in a normal population a difference in the occurrence of beta hemolytic streptococci on the throat and on anterior gingiva might be anticipated and that the gingival or "dangerous" carrier would appear some-

what less often than the throat carrier. Examination of persons for the presence of beta hemolytic streptococci should include cultures made from the gingiva as well as from the throat.

The authors wish to thank Dr. Joaquin Munoz and Dr. Milan Novak of the Department of Bacteriology for assistance and advice during this study.

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CHEMICAL STUDIES IN PERIODONTAL DISEASE

VI. The Glycogen Content of Gingival Tissue in Alloxan Diabetes

B. COHEN AND L. S. FOSDICK

Chemistry Department, Northwestern University Dental School, Chicago, Ill.

IN THE fifth study of this series[1] it was revealed that the glycogen content of pathological and of traumatized gingival tissue is significantly lower than that of apparently normal gingivae. The present study was undertaken in an effort to ascertain whether or not gingival glycogen content underwent alteration in the early stages of diabetes produced by intravenous administration of alloxan.

Diabetes mellitus has been widely cited as an instance of systemic disorder leading to periodontal disturbance.[2-8] While general agreement as to its significance in this regard has not been reached, a survey of literature on the subject suggests that a large majority of authors regard periodontal disease as being present more frequently in diabetic than nondiabetic subjects. The lack of evidence to show that diabetes and periodontal disease are invariably related is exemplified by the work of Glickman[9] who demonstrated that the nature and incidence of histological changes in the gingivae are not peculiarly altered in rats rendered diabetic by the use of alloxan. This latter report is substantiated by the lack of positive findings recorded in a survey of a large group of human diabetics.[10]

Thus, although the consensus favors the view that periodontal disease is more common among diabetics than nondiabetics, considerable evidence is available to show that pathological involvement of the interesting tissues of the teeth is not an invariable concomitant of the diabetic syndrome. For this reason it is the opinion of the present authors that terms such as "diabetic gingivitis" are misnomers, since they imply that gingival lesions are a specific entity inevitably associated with the diabetic state. No evidence is available to show that pathological changes in the gingivae arise as a direct result of diabetes. It would seem probable, however, that diabetics may be predisposed to gingival disorders as suggested in the hypothesis that diabetes is a conditioning factor in the etiology of periodontal disease, rendering the gingival tissues more susceptible to damage and more vulnerable to superimposed infections. In other words, the role of diabetes in the etiology of periodontal disease is probably not that of a direct agent, but rather of a catalyst, as it were, to the exciting factor.

The findings of Richardson,[11] which demonstrate that proteolysis is inhibited in the presence of glycogen, provoked the supposition proffered in an earlier report,[1] to wit, that glycogen might act as a protective agent in such conditions as tend to instigate the degradation of tissue protein. That periodontal disease is such a condition seems certain, and confirmatory evidence of this fact has been

The work was submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Northwestern University, and was partially supported by Colgate Palmolive Peet Company and the Lambert Pharmacal Company.

Received for publication, April 18, 1949.

recorded.[12, 13] The present investigation was undertaken to pursue the possibility that the predisposition of diabetic subjects to periodontal disease might be associated with alteration in the glycogen content of the gingivae. No attempt was made to produce diabetes of long standing; instead, the experiment was designed to examine the early reaction, if any, to the metabolic disorder induced. Lackey, Bunde, Gill, and Harris[14] and other investigators, have demonstrated that the production of experimental diabetes results in a diminution of liver glycogen content; liver glycogen was therefore estimated concurrently with that of the gingivae, as a check on the effectiveness of the diabetogenic agent administered and the reliability of the analytical method employed.

EXPERIMENTAL PROCEDURE

It was originally intended that Syrian hamsters be used for this study. For several reasons, however, these animals proved unsuitable. In the first place, the absence of accessible veins suitable for venepuncture rendered the administration of intravenous alloxan extremely difficult; moreover, the collection of samples for blood glucose estimations was equally complicated since the blood of the hamster coagulates within a few seconds of breaking surface. In addition, these animals lack the tractability desirable for handling them without recourse to anesthesia. Finally, the amount of gingival tissue which could be recovered from the hamster was so minute as to render its analysis impracticable.

Some experience of these difficulties led to the substitution of rabbits for hamsters. The results recorded here are derived from experiments conducted on fourteen animals, seven of which are kept for controls and seven of which were subjected to intravenous injection of a 5 per cent aqueous solution of the monohydrate of alloxan. A detailed account of dosages and mortality among animals not included in this report has been recorded elsewhere.[15]

After the injection, the animals were kept under careful observation, and precautions were taken to ensure that they were constantly supplied with food and water. Attainment of the diabetic state was gauged by blood glucose estimations (resting, not fasting) alone. For corroboration, liver glycogen content was estimated concurrently with the gingival analysis of the animal sacrificed. Comparison of fasting blood glucose levels was originally intended. However, three of the first five animals treated succumbed when subjected to fasting. To avoid this hazard, and because the degree of hyperglycemia produced by alloxan's destruction of the pancreatic islets is so unmistakably pronounced, a comparison of normal bloods was deemed sufficient for the purposes of this study. The mean blood glucose level recorded for the normal resting animal was 110.6 milligrams per 100 cubic centimeters. (Range 102.5 to 129.5.) That of the animals treated with alloxan was 577.7 milligrams per 100 cubic centimeter. (Range 465 to 763 mg./100 c.c.)

The following method was used for the estimation of the blood glucose level: (a) 0.1 c.c. of blood was laked in a test tube containing 2 c.c. of distilled water. (b) The sample was deproteinized by the addition of 0.2 c.c. 7 per cent $CuSO_4 \cdot 5\ H_2O$; after shaking, 0.2 c.c. of 10 per cent sodium tungstate were added, the tubes were stoppered and again shaken well; the samples were then allowed to stand for ten minutes. (c) The samples were next centrifuged for 7 minutes,

after which the supernatant liquid was filtered through a micro-Buchner funnel using Whatman's No. 42 filter paper. (d) One cubic centimeter of the filtrate was then transferred to a 25-c.c. Pyrex volumetric flask and blood glucose estimated according to the method of Farmer,[16] a description of which is given below in the account of methods used for the analysis of glycogen.

Within two hours of taking samples for the final blood glucose estimation, the animal was sacrificed. The method used was devised in an effort to combat the instability of body glycogen, which would be rapidly depleted by death struggles as well as by enzymic changes post-mortem. A container of capacity sufficient to accommodate the animal was lined on floor and walls with slabs of dry ice. A few cubic centimeters of water were sprinkled into this chamber resulting in the formation of a dense atmosphere of carbon dioxide. The animal, previously soothed to ensure a relaxed state, was quickly placed into the chamber which was then covered with a final slab of dry ice and the whole covered with wrapping paper to preclude the entrance of air. Death supervened very rapidly; the absence of struggling was noteworthy and limited to faint asphyxial gasps. In this manner loss of glycogen due to body exertion was reduced to a minimum. Loss of glycogen through enzyme reactions post-mortem was also minimized since the tissues were fixed in a frozen state. The animal was removed from the sacrificial chamber within fifteen minutes. Dry ice was kept in contact with the mouth while liver samples were taken. The abdominal cavity was opened and two samples were removed from the inferior border of the right hepatic lobe as expeditiously as possible. These samples were placed in thin-walled glass containers resting on dry ice. Two samples of gingival tissues were then removed. In the lower jaw, gingival tissue was taken from the labial and lingual aspects of the two lower incisors; a fine blade was passed down the gingival crevice which is considerably deeper in the rabbit than in the human species and the gingivae freed by rapid excision. The sample was stored as in the case of liver tissues. The maxillary gingival tissue, taken from the labial and palatal aspects of the upper incisors, differed morphologically from the mandibular counterpart. Very little tissue could be recovered from the labial surface of the upper incisors as the gingivae in this area merge with the frenum and the muscle insertions of the bilateral upper lip; most of the tissue was recovered from the palatal side and comprised a somewhat flabby fibrous tissue, not closely adherent to the bone, and comparable in nature, although not in site, to enlarged palatal rugae in the human being.

The excised tissues were transferred directly from the refrigerated glass containers into individual 15-c.c. conical centrifuge tubes, each containing 1 c.c. 30 per cent potassium hydroxide, and each previously weighed. The tube containing the tissue was then reweighed and the weight of the tissue thus determined. The tubes were then loosely stoppered and placed in a steam bath for thirty minutes. In this manner the tissue was digested to a clear solution; 1.5 c.c. of 95 per cent ethyl alcohol were then added and boiled for one minute. The tubes were then cooled under running water and a flocculent precipitate of glycogen resulted. At this state the tubes were left in a refrigerator overnight. According to Berg, Lundquist, Schram, and Fosdick[1] the samples may be so stored

"for as long as ten days without appreciable deterioration." Continuing the procedure, the precipitate was centrifuged at 3000 r.p.m. for thirty minutes and then drained. Thereafter 2 c.c. of 95 per cent ethyl alcohol were added and the tubes again centrifuged at the same speed as previously for the same length of time before being thoroughly drained once more. The next stage comprised the hydrolysis of the glycogen in the precipitate to glucose. Initially one microdrop of 50 per cent hydrochloric acid was added, followed by 2 c.c. of 1 Normal hydrochloric acid. The tubes were then fitted with air condensers and boiled in a steam bath for two hours. The resultant acid solution of glucose was cooled and analyzed for glucose content according to the method of Farmer.[16] This method was used in the estimation of blood glucose levels (vide supra) according to the following procedure: 1 c.c. of the solution was withdrawn by pipette and introduced into a 25-c.c. Pyrex volumetric flask. Three drops of an indicator (phenolphthalein) were added and the solution made alkaline by gradual addition of 1 Normal sodium hydroxide. One cubic centimeter of a copper sulfate reducing reagent was added. The flasks which were covered with tinfoil caps were then boiled in a water bath for precisely ten minutes, after which time they were cooled under running water and 5 c.c. of phosphotungstic acid reagent were added. Color was allowed to develop before diluting to 25 c.c. volume with distilled water. The intensity of blue color developed is an index of the glucose content. This was read off on a Coleman Universal Spectrophotometer at a wavelength of 725 millimicrons using a red filter (PC 5). The reading, expressed in percentage of light transmission, was converted to gamma of glucose per cubic centimeter of the solution by reference to a graph previously plotted from the light transmissions of known samples of pure glycogen. From the weight of tissue previously deduced the percentage of glycogen in the tissue could then be computed.

Certain modifications of this method were found to be necessary in the case of liver analysis in order to ensure that the ultimate color produced would be within readable range on the spectrophotometer. For this reason hydrolysis was effected with 5 c.c. of hydrochloric acid instead of the 2 c.c. amount related above, thereby increasing the dilution of the acid glucose solution from 1:2 to 1:5. In addition, in the case of liver tissue taken from control animals, it was found advisable to dilute the final solution up to 100 c.c. instead of 25 c.c. These increased dilutions were allowed for in computing the quantity of glycogen present.

RESULTS

TABLE I
MEAN TISSUE GLYCOGEN CONTENT OF CONTROL ANIMALS

RABBIT NUMBER	LIVER (%)	GINGIVA (%)
1	4.625	0.107
2	4.670	0.080
3	10.780	0.668
4	4.490	0.065
5	6.980	0.097
6	2.940	0.022
7	9.600	0.099

TABLE II
MEAN TISSUE GLYCOGEN CONTENT OF ANIMALS TREATED

RABBIT NUMBER	LIVER (%)	GINGIVA (%)
8	0.820	0.026
9	0.860	0.024
10	0.730	0.026
11	0.940	0.013
12	0.147	0.023
13	1.280	0.030
14	1.420	0.019

TABLE III
COMPARISON BETWEEN CONTROLS AND TREATED ANIMALS

	CONTROLS	TREATED ANIMALS
Mean blood glucose level before sacrifice	116.1	577.7
Mean glycogen content of liver tissue	6.296%	0.885%
Mean glycogen content of gingival tissue	0.163%	0.023%

TABLE IV
STATISTICAL ANALYSIS OF RESULTS

	LIVER	GINGIVAE
t Ratio	26.77	8.84
P	.01	.01

DISCUSSION

Table I reveals that the liver glycogen content of control animals varied from means of 2.94 per cent to 10.78 per cent, with an over-all mean of 6.30 per cent. According to Best and Taylor[17] the percentage of glycogen present in the liver may vary from 0.2 to 10. The comparable figure given by Hawk, Oser, and Summerson,[18] is "a trace to upwards of 8 per cent of the weight of the organ." In the work of Lackey, Bunde, Gill, and Harris[14] glycogen content of liver, heart, and skeletal muscle of rats was estimated. The liver glycogen was reported to be 3.056 per cent in control animals. The difference between this figure and that recorded in Table I may be due to a difference between rats and rabbits; but since the values given in Table I are generally higher than the estimates of other investigators as well, it is possible that the disparity may be due to the fact that loss of glycogen was cut down to a minimum by the method of sacrifice employed in this study.

No comparison to the gingival findings could be disclosed by a study of the literature. The only other chemical analysis of gingival glycogen noted[1] was carried out on human biopsy material; in that investigation the content of normal tissue was found to be 0.224 per cent.

A comparison of the respective glycogen values of liver and gingivae in Table I appears to indicate some degree of interrelation. It will be noted, for example, that Rabbit No. 6 which showed the lowest gingival glycogen among the controls also showed the lowest liver glycogen content; and Rabbit No. 3 which possessed most liver glycogen of the animals in this series also demonstrated the highest gingival values. Statistical treatment of these data reveal that there is a 95 per cent correlation between the two sets of readings.

Table II reveals no such correlation in the case of the treated animals. Both gingival and liver values are markedly decreased, but there is no evidence that shows this decrease takes place to the same extent in the two tissues in individual animals.

Table III illustrates the degree to which the administration of alloxan depletes the tissues analyzed of the glycogen they contain. The mean blood glucose values are included to demonstrate the diabetic response. The readings from which these figures are derived are given in full elsewhere.[15] It will be noted that in the control group as well as in the treated group of animals, the proportion of gingival glycogen to liver glycogen was approximately one to thirty-eight; and that the ratio of control to treated glycogen content was almost identical in the case of the two tissues analyzed (approximately 7:1). It is reiterated therefore that whereas there is a considerable degree of correlation between liver and gingival glycogen in the controls, statistical analysis reveals that there is none whatever in the case of the treated animals. Thus, although a mutual ratio of attractive possibilities appears to exist, in point of fact any such assumption is invalid. The significance of this phenomenon escapes assessment at the present time.

From Table IV it is seen that the results of this experiment demonstrate one fact incontrovertibly; to wit, that the production of an experimental diabetic state results in the depletion of gingival glycogen. To this extent they support by chemical evidence the important principle that metabolic disorder can exert oral effects. Estimation of the t ratio demonstrates that the differences between tissues in the two groups of animals are significant and that P, or the probability of chance, is much less than one in one hundred.

When these findings are viewed in conjunction with the observations of other authors, certain interesting possibilities are brought to mind. As has been stated previously, the weight of available evidence indicates that periodontal lesions are more common in diabetic than nondiabetic subjects; second, the work of Berg, Lundquist, Schram, and Fosdick[1] and Richardson[11] has led to the suggestion that tissue glycogen by inhibiting the breakdown of protein may serve a protective function. It would seem within the bounds of possibility, therefore, that the manner in which diabetes may predispose to periodontal pathogenesis is by the depletion of protective substance, namely, glycogen from the periodontal tissues. In this event, the findings of Ziskin, Loughlin, and Siegel[19] are worthy of close study, for these workers have reported that an increased deposition of glycogen in the gingivae of Rhesus monkeys can be effected by the topical application of insulin.

Correlation of these findings leads to the hypothesis that periodontal disturbances should be alleviated by topical application of insulin to the gingivae; the possibility of prophylactic rather than palliative treatment would of course arise.

Although this surmise has attractive therapeutic possibilities, it should be stressed that further experimental evidence requires to be accumulated before the treatment mooted can justifiably be put to the test of clinical application. In the first place, it is essential that experimental proof be provided to ascertain

that topical application of insulin would be confined in its effects to the tissues to which it is applied, lest the use of such therapy provoke untoward body-wide effects. Second, it is worthy of note that the findings of Ziskin, Loughlin and Siegel[19] are based on histological evidence, the accuracy of which is not deprecated when it is suggested that their value would be enhanced if the same findings could be repeated by chemical investigation. It is therefore intended to conduct further experiments along these lines. If such experiments were to divulge data confirming the suppositions mentioned previously, and if blood glucose estimations were to offer assurance that topical application of insulin exerted no systemic effects, then and only then would it be justifiable to admit this procedure to the realm of practical therapeutics. Moreover, it is reasonable to assume that its use would not be confined to purely dental spheres. In analogous manner this topical application of insulin might serve to assist the healing of injuries elsewhere on the body surface if the depletion of cutaneous glycogen is depriving the tissues of a natural protective agent; delayed healing of trivial injuries has long been recognized as a feature of the diabetic state. Finally it is suggested that the glycogen content of gingivae in other conditions which appear to predispose to periodontal disease would merit investigation; the particular association of insulin with the treatment of diabetes should not be allowed to conceal the possibility that the drug might be used to advantage as a topical medicament in nondiabetic conditions—always providing that experimental evidence can be adduced to attest to its safety as a therapeutic agent.

SUMMARY

The glycogen content of gingival tissue in animals suffering from alloxan diabetes has been determined. There is a statistically significant decrease in gingival glycogen under these conditions.

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CHEMICAL STUDIES IN PERIODONTAL DISEASE

VIII. Gingival Glycogen Concentration in Experimental Occlusal Trauma

C. A. OSTROM,*† W. G. SKILLEN, AND L. S. FOSDICK

The Clinics and Laboratories, Northwestern University Dental School, Chicago, Ill.

OCCLUSAL trauma has long been considered one of the etiologic factors in periodontal disease. Miller[1] lists occlusal trauma as a primary inciting factor of periodontal disease and Box[2] lists increasing depth of the pocket and crevicular pus formation as diagnostic signs of occlusal trauma. Another school of thought includes Becks[3] who holds that occlusal trauma is not necessarily the primary etiologic factor, but that it may be one of the local factors which precipitate the disease when the systemic predisposing condition is present. These two concepts are still widely accepted in spite of the fact that experimental evidence is lacking and in spite of the experimental findings of Gottlieb and Orban.[4] Orban[5] stated that in his opinion occlusal trauma and its effects is a separate entity from and not a causal agent of periodontitis and that the two are found together in 90 per cent of periodontitis cases is a coincidence brought about by the relatively high incidence of both pathological conditions.

It has been shown that traumatized tissue will have a reduced glycogen concentration.[6, 7, 8] Further, Berg, Lundquist, Schram, and Fosdick[8] reported altered gingival glycogen concentrations both in periodontoclasia and in cases of experimental trauma of gingivae. From this basis it is suggested that experimental occlusal trauma might alter the gingival glycogen concentration.

This study was undertaken to test the hypothesis that occlusal trauma is a primary etiologic factor in the production of periodontal disease. This hypothesis would receive substantiation if experimental occlusal trauma were shown to alter the gingival glycogen concentration.

PROCEDURE

Vitallium splints‡ were applied to fifteen dogs and were designed to produce abnormal occlusal stress acting on normal periodontal tissues. This is what Box[2] calls traumatogenic occlusion. These splints were made to fit the mandibular teeth of dogs and extended from canine to canine tooth; were cemented to place and then further secured by means of rivets through the incisal one-third of the two canines; the incisal height was one-fourth of an inch; only the maxillary incisors could be occluded; and the incisal edge of the splint was angled at about forty-five degrees to produce marked lateral stress injury.

This work was supported by grants from the Colgate Palmolive Peet Company, Jersey City, N. J., and the Lambert Pharmacal Company, St. Louis, Mo.

Received for publication, April 18, 1940.

*Commander, DC, USN.

†The opinions and assertions contained in this article are the opinions and assertions of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.—Article 113, U.S. Navy Regulations

‡Courtesy of the Kennedy Co. and the Austenal Co., Chicago, Ill.

After these splints had been in place for two weeks, two samples of the gingivae were taken from the maxillary incisal region of thirteen of these dogs for determination of the glycogen concentration and the remaining two dogs were used to provide samples for histologic study of the effects of the splints. Two samples were taken from each of the twelve normal dogs to determine the normal gingival glycogen concentration.

Since it was found that the gingivae of the dogs bled much more profusely than was experienced with human gingivae[9] and since it has been reported that bleeding will alter the glycogen concentration of a sample,[6, 7] it was necessary to devise a method for taking the samples which would reduce the bleeding but which would not effect the gingival glycogen content. For these reasons, the dog was carried to the surgical stage of anesthesia with intravenous Nembutal and then the dog's entire head was placed in a specially prepared box where the mouth was packed and the entire head was buried in crushed dry ice. Of course the dog died of carbon dioxide asphyxiation, but it is thought that the gingivae were frozen before death occurred.

Early tests showed that dogs have an extremely low gingival glycogen concentration and, therefore, to make for greater accuracy in the quantitative analysis, samples of 80 to 100 mg. were taken in all cases.

The method of analysis was the same as that described in a previous report by the authors.[9]

Statistical analysis of the gingival glycogen concentration of normal dogs as compared to the splinted dogs should indicate whether experimental occlusal trauma causes an altered gingival glycogen content.

The samples for histological study were fixed, decalcified, embedded in celloidin, sliced, and stained by hematoxylin-eosin.

RESULTS AND DISCUSSION

TABLE I

	NORMAL	TRAUMATIZED
Number	24	26
Mean glycogen content	.039 mg. %	.038 mg. %
Standard deviation	.046	.031
Coefficient of variation	118%	81.6%
Range with 95 per cent Confidence limits	0 to .134 mg.%	0 to .102 mg. %
Per cent within range	92%	93%
Standard error of difference between means	.0110	
t—ratio	.09	
Significance	no	

The statistical analysis of the glycogen concentration of normal gingivae as compared to the gingivae of the dogs with experimental occlusal trauma showed no significant difference between the two. Clinical examination during the two-week period showed no apparent change in the color or texture of the gingivae, no periodontal pockets, and no loosening of the teeth.

Histological study bore this out inasmuch as no hyperemia or signs of inflammation existed. Otherwise, the periodontal membrane changes were similar

Fig. 1.—Labial tipping. Labial side, on which pressure is being exerted, is on right; lingual side, under tension, is at left.

Fig. 2.

Fig. 3.

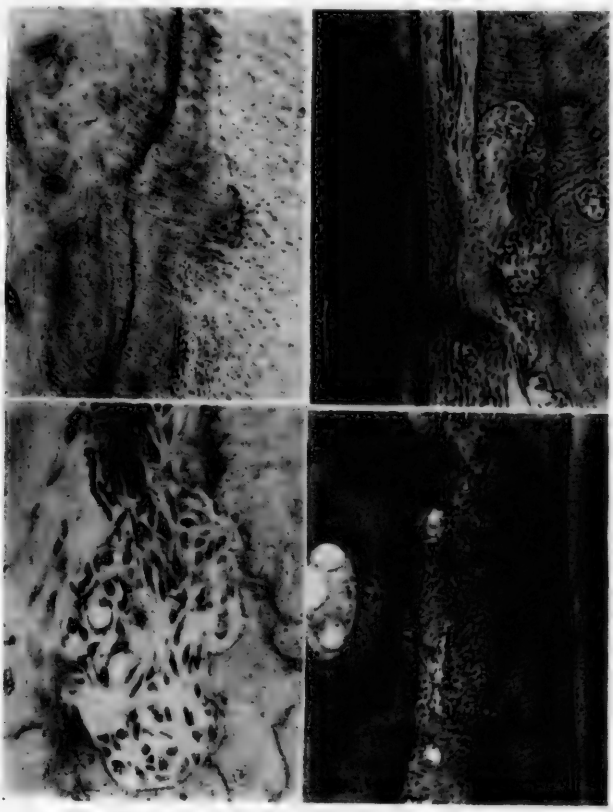

Fig. 4.

Fig. 5.

Fig. 2.—Lingual (tension) side of Fig. 1. Note new bone formation.

Fig. 3.—Labial (pressure) side of Fig. 1. Note compressed periodontal membrane and undermining bone resorption.

Fig. 4.—Undermining bone resorption (Fig. 3) showing Howship's lacuna.

Fig. 5.—Apical lingual pressure area from Fig. 1 showing congested vessels in periodontal membrane.

to the findings of Gottlieb, Orban and Diamond.[4, 10] Fig. 1 is a seven-power magnification which shows that the maxillary incisor has been tipped labially; on the tension side the periodontal membrane width is abnormally large; and on the pressure side the periodontal membrane width has been reduced by approximation of the tooth to the wall of the socket. Fig. 2, which is a low-power view of the lingual area near the crest of the alveolus, shows considerable new bone formation and taut periodontal membrane on the tension side. New cementum could be recognized in this area but it is not shown on the photomicrograph. Fig. 3 is a low-power view of the labial side near the crest of the alveolus and shows crushed periodontal membrane. Adjacent to this area is considerable undermining resorption of the bone. Fig. 4 is a high-power view of this osteoclastic activity. Fig. 5 is a low-power view of a pressure area near the apex on the lingual side and shows relaxed periodontal membrane and more or less circular vessels and interstitial spaces. Hence, all of these histologic findings provide evidence that pressure changes in the supporting tissues were obtained. Whether these pressure changes should be considered evidence that occlusal trauma was obtained needs further consideration. All of these histological changes can be found in the supporting tissues of unerupted teeth as well as in mouths showing no clinical evidence of pathological changes due to occlusal trauma.

The period of trauma, if it may be called such, might also be subject to criticism. In this study the teeth were subjected to the experimental occlusal trauma for only two weeks, whereas it is conceivable that a much longer period might have produced an entirely different picture both in the clinical and histological findings as well as in the gingival glycogen content.

CONCLUSIONS

This study has shown that splints did produce pressure changes which might be expected from occlusal trauma and that this experimental occlusal trauma did not alter the gingival glycogen content. Hence, this study fails to support the hypothesis that occlusal trauma is a primary etiologic factor in periodontal disease.

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THE EFFECT OF CERTAIN LUBRICATING AGENTS AND COARSE FOODS UPON THE CORNIFICATION OF THE ORAL MUCOSA OF THE WHITE RAT

ABRAHAM PERLMAN

Department of Anatomy, College of Dentistry, and the Graduate School of Arts and Science, New York University, New York, N. Y.

THE gingiva during mastication is exposed to heavy mechanical stresses, and the thick cornification provides a form of protection from injury both to the underlying tissues and to the epithelial attachment of the tooth.[3] It has been shown that this cornification may be obtained by massage either through the correct use of the toothbrush or through the mastication of coarse foods.[4]

Butcher[1] found that local application of olive oil to the skin of the white rat prevents dehydration of the stratum corneum. The present investigation has been undertaken to study what effects, if any, the addition of fat to a coarse diet would have upon the cornification of the oral mucosa of the white rat.

MATERIALS AND METHODS

Albino rats averaging 125 grams were placed on the following diets: (a) Purina Chow, which is an adequate nutriment consisting of meat, wheat germ oil, dried skim milk, cod liver oil, barley malt, molasses, oat and corn cereal, wheat bran, and iodized salt. Purina Chow on analysis is found to contain not more than 3 per cent fat; (b) dried whole kernel corn; (c) yellow corn meal; (d) dried whole kernel corn plus 10 per cent vegetable oil*; and (e) yellow corn meal plus 10 per cent vegetable oil. Where vegetable oil was added to the diet, melted fat was poured into a jar of corn and the jar shaken vigorously for several minutes.

The animals were sacrificed four and six weeks after being fed their respective diets. The mucous membrane was carefully stripped from the following areas: (a) the hard palate; (b) a four millimeter rectangle taken buccally from the mesial surface of the first mandibular molar to the distal surface of the third mandibular molar; and (c) a similar rectangular area from the maxillary region. All tissues were fixed either in Bouin's or Zenker's fluid, and they were stained with hematoxylin and eosin.

RESULTS

The epithelium of the mandibular gingiva from the rats fed whole kernel corn and corn meal did not differ essentially in structure from each other.

The stratum corneum (Figs. 1 and 3) was compact and compressed, being 40 to 50 μ in thickness. Cell outlines were nowhere visible. The surface was regular with little desquamation, the entire corneum staining deeply with eosin.

Received for publication, April 29, 1949.

*As contained in Crisco which is a commercial product consisting of hydrogenated vegetable oils.

The corneum was well demarcated from the underlying stratum granulosum. The cell boundaries were not discernible even in the layers immediately adjacent to the stratum granulosum. The stratum spinosum consisted of several layers of very small cells. Cell outlines were not visible, and the cytoplasm was finely granular and slightly basophilic.

The epithelium of the mandibular gingiva from the rats fed whole kernel corn plus vegetable oil, and yellow corn meal plus vegetable oil differed neither from each other nor greatly from rats fed Purina Chow (Figs. 2, 4, and 5). The stratum corneum was 10 μ thicker on the average than that seen in the corn-fed animals and was composed of acidophilic nonnucleated cells with serrated cell membranes. In contrast to the corn-fed animals, cell outlines were quite distinct even in the more superficial layers of the corneum. The stratum spinosum from animals fed fat supplement consisted of large oval or slightly polygonal cells containing a clear and lightly staining cytoplasm with very distinct cell boundaries. This contrasted sharply with the small granular cells with indistinct cell outlines which were seen in the animals fed corn alone.

The epithelium of the maxillary gingiva from the rats of all experimental groups differed in no significant manner. A picture quite similar to that seen in the mandibular gingiva of the animals maintained on Purina Chow was noted.

The epithelium from the hard palate of all experimental groups varied little. The entire epithelium was compressed. The stratum corneum, 30-40 μ in thickness, was a heavily keratinized structure wherein cell outlines were nowhere evident, and the over-all appearance was that of several layers of cornified cells so closely packed as to appear almost homogeneous.

DISCUSSION

In these studies it was seen that the stratum spinosum as well as the stratum corneum was affected by the mastication of coarse diets (kernel corn and corn meal). The effect was of a compressive nature, resulting in very flat, densely packed cells in the corneum and slightly granular cells between which cell boundaries could not easily be discerned in the spinosum.

When 10 per cent vegetable oil was added to the corn diets, there was less tendency toward packing and compression of the epithelium; the cells of the stratum spinosum were large, few in number, contained much more cytoplasm, and appeared much less dehydrated.

These findings would tend to confirm those of Butcher[1] who found the local application of olive oil to retard the dehydration of the skin of the white rat. The cells of the corneum appeared to "pile up" without sloughing off. When the oil treatment was discontinued, the surface layers were desquamated, the epithelium returned to its normal thickness, and a well-keratinized corneum redeveloped.

Although the exact mode of action of the vegetable oil must remain conjecture, several points should be examined. In Butcher's study[1] olive oil was locally applied to the skin. The marked similarity between the effects produced and those observed in this study, coupled with the finding that only the man-

dibular gingiva was affected, would tend to favor a theory of local action. What factor, then, common to olive oil and vegetable oil might be the mediator of this local action? A most logical conclusion would appear to be, in this writer's opinion, the physical property common to both fats, that is, their lubricating action. In other words, any palatable fat or oil, whether a true lipid or not, would be expected to give similar results.

Fig. 1. Fig. 2.

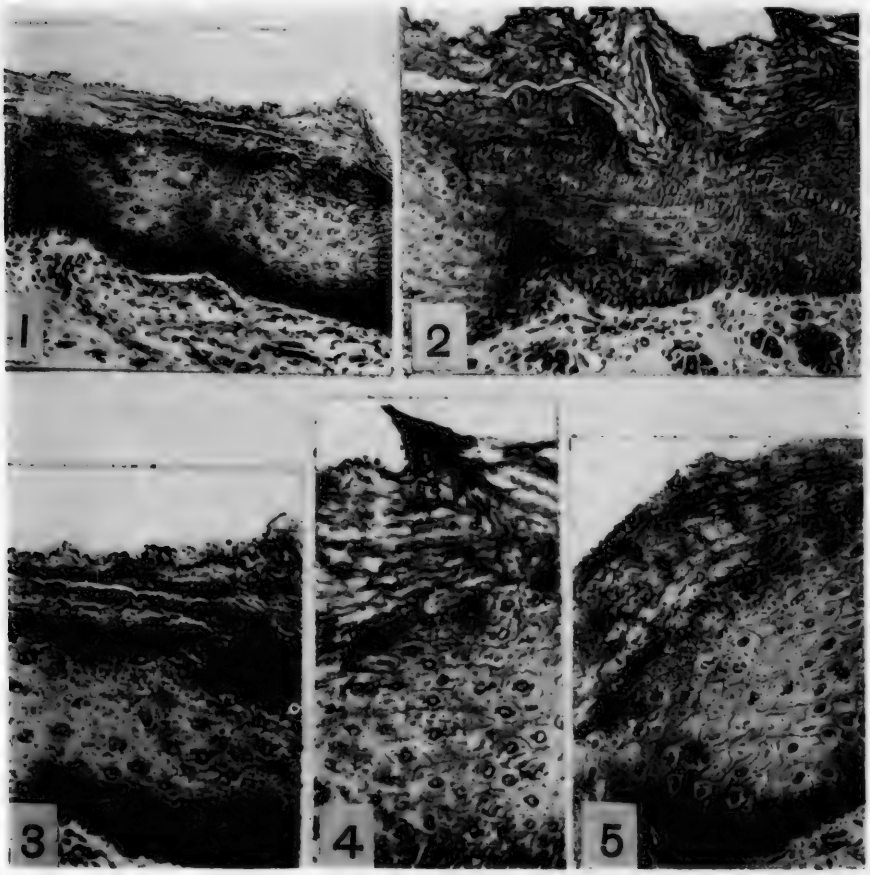

Fig. 3. Fig. 4. Fig. 5.

Fig. 1.—Mucous membrane from mandibular region of rats fed kernel corn and corn meal. The stratum corneum shows a clear demarcation from underlying stratum granulosum. Cell outlines are not visible even in deeper layers of the corneum. ×130.

Fig. 2.—Mucous membrane from mandibular region of rats fed kernel corn and corn meal supplemented with 10 per cent vegetable oil. Cell outlines are apparent even in most superficial layers of corneum. Stratum granulosum blends gradually into corneum. ×130.

Fig. 3.—Mucous membrane from mandibular region of rats fed kernel corn and corn meal. Stratum corneum consists of flattened, compressed cells with no apparent cell boundaries. Stratum spinosum is composed of small, finely grandular cells with indistinct cell membranes. ×220.

Figs. 4 and 5.—Mucous membrane from mandibular region of rats fed kernel corn and corn meal supplemented with 10 per cent vegetable oil. Polygonal cells with distinct wavy cell outlines make up stratum corneum. Spinosum contains large oval or slightly polygonal cells filled with clear, lightly stained cytoplasm. ×220.

As a concrete example, the substitution of liquid petrolatum for vegetable oil should give entirely analogous results. Liquid petrolatum (U.S.P.) or mineral oil is not a lipid, is not acted upon by digestive ferments, and is not absorbed.[2] By substituting mineral oil for vegetable oil we may at once determine whether the action is a local one and whether or not it is due to any nutritional factor contained in the fat. This substitution was made and the results were identical with those of the vegetable oil-fed animals.

SUMMARY

1. The gingival mucosa of the white rat maintained on a diet of Purina Chow shows a stratum corneum consisting of several layers of nonnucleated polygonal cells showing distinct cell boundaries, the deeper layers of which show no marked differentiation from the underlying stratum granulosum.

2. Rats maintained on a diet of kernel corn or corn meal have a stratum corneum consisting of several layers of compressed, flattened cells between which cell membranes are not apparent and whose deeper layers show a clear line of demarcation from the underlying stratum granulosum. The stratum spinosum is composed of small cells with finely granular basophilic cytoplasm with barely discernible cell outlines.

3. The above effects are more readily observable in the gingiva of the mandible; the maxillary gingiva in all cases remaining identical.

4. The addition of 10 per cent vegetable oil to the corn diets acts to antagonize their dehydrating and compressing effects upon the mandibular gingiva. The stratum corneum of these animals consists of large polygonal cells with distinct cell outlines, and the stratum spinosum is composed of voluminous cells with deeply staining centrally placed nuclei.

5. The action of vegetable oil is a local one and is probably due to its lubricating property, for mineral oil produces entirely analogous results. The oils apparently prevent dehydration, and the cells remain soft and full of cytoplasm.

6. The epithelium of the hard palate, already dehydrated, shows no tendency toward changing in response to the mastication of a coarse diet, and also shows no tendency toward alteration when subjected to a fat-supplemented diet.

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A DIETARY ASPECT OF CALCULUS

B. F. GURNEY, M.S., AND JOHN H. HUSCHART, B.S.

From the Department of Research of the Loyola University School of Dentistry, and Chicago College of Dental Surgery, Chicago, Ill.

MANY investigations have been made to establish the relationships between the concentration of salivary components and the presence of caries. In recent years considerable effort has been expanded in attempts to correlate salivary composition with the presence or absence of calculus.

Since calculus is composed mainly of calcium and phosphorus as the phosphate salt, it follows from various chemical laws that there must be a definite chemical relation between calculus and the calcium and phosphate ions in the surrounding saliva. This relationship has been elucidated by previous workers.[1] Suffice it to say here that when the product of the calcium- and phosphate-ion concentrations exceeds a certain value in a given sample of saliva, then there is a definite tendency toward the production of calculus by that saliva.

Most of the investigations in this problem of calculus and salivary composition have been made upon saliva from fasting individuals. Conclusions from such investigations must of course apply to salivary conditions existing primarily under fasting conditions. Now most individuals have a habit of eating, and since food usually contains appreciable amounts of calcium and phosphorus in soluble form, it would seem logical that the ingestion of food might have an effect on the excretion of calcium and phosphate ions in the saliva, which in turn might increase or decrease the tendency of saliva to deposit calculus.

This investigation therefore was undertaken to determine if dietary intake effects the calcium- and phosphate-ion concentrations in the saliva. For purposes of discrimination it was deemed necessary to know the fluctuations (if any) in salivary compositions that take place with the ingestion of $Ca_3(PO_4)_2$ fortified solutions. This could then be compared with changes arising from the ingestion of a fortified test meal. Only the first part of this work, together with chemical and clinical results showing the relation of such changes to calculus formation, is reported in this paper.

EXPERIMENTAL

Ten normal, healthy, young adult male dental students were chosen and investigated under given conditions. Subjects reported at all times without breakfast. Following collection of the first saliva sample, they ingested various items and water. Then at ensuing 15-minute intervals for the first hour and 30-minute intervals for the second hour, saliva samples were collected and treated as follows.

Received for publication, March 30, 1949.

A pH determination on a small fraction of the saliva was made immediately. The remainder of the sample was separated from its proteins by addition of trichloracetic acid and by centrifugation. The resultant supernatant liquid was divided and calcium and phosphate analysis made on the respective fractions.

All saliva samples were obtained from the subjects by having them chew unflavored paraffin. All analytical technics were checked against prepared standard solutions. Phosphate was determined colorimetrically according to the method of Sumner and with the aid of a Lumetron photoelectric colorimeter. Calcium was determined by the usual microvolumetric method. All subjects were given complete oral examinations by the clinic, each individual being examined by two and usually three members of the clinical faculty.

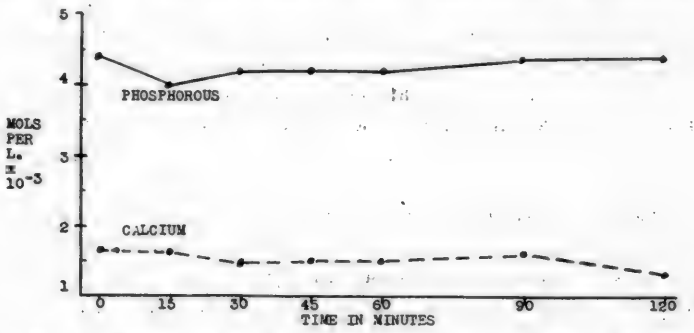

Fig. 1.—Salivary Ca and P concentrations. Part II, Subject B, Trial 1.

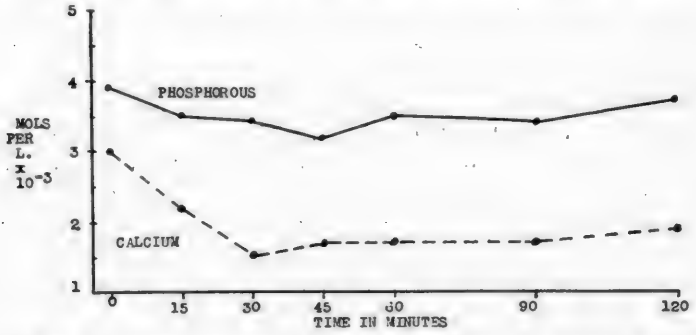

Fig. 2.—Salivary Ca and P concentrations. Part II, Subject B, Trial 2.

DISCUSSION

During Trial 1 the subjects ingested 250 c.c. of water, nothing else. The purpose here was to test the effect of water alone on the concentrations of the ions under investigation. Results characteristic of this series of tests are shown in Fig. 1.

Attention is directed not so much to the concentration values themselves, but rather to the trend that these values exhibit for herein may lie the answer to some of the problems connected with calculus formation.

During Trial 1 it might have been expected that the ion concentrations would decrease with time. Previous investigations[2] as well as our own show that in stimulated saliva the concentrations would have decreased anyway. This follows simply from the fact that during the collection of stimulated saliva the increase in calcium and phosphate ions excreted does not match the increase in liquid excreted; hence the ion concentrations must fall. The important question here is whether or not the intake of 250 c.c. of water produces a change in salivary ion concentrations which is discernible from that which normally takes place. Results from these ten individuals lead to the conclusion that at least these subjects exhibited no appreciable differences.

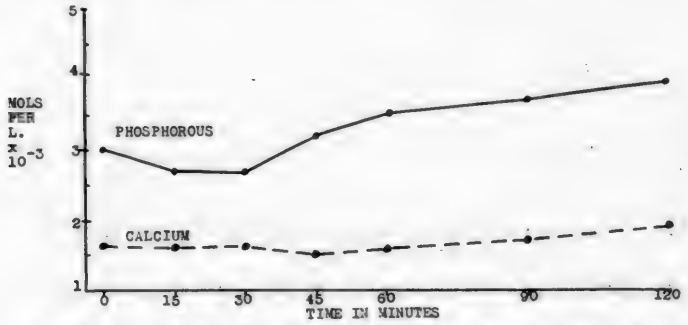

Fig. 3.—Salivary Ca and P concentrations. Part II, Subject B, Trial 3.

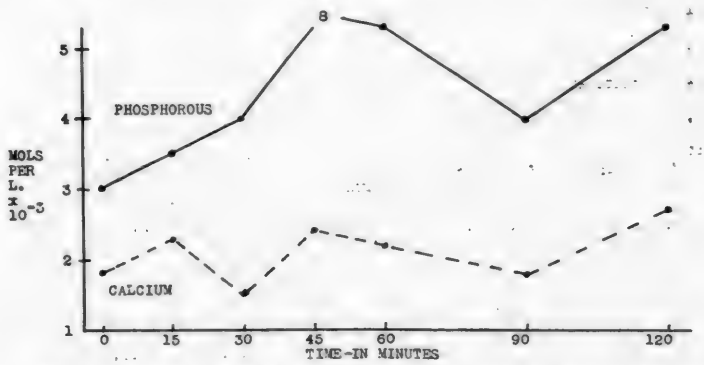

Fig. 4.—Salivary Ca and P concentrations. Part II, Subject F, Trial 3.

Fig. 2 shows the results of ingestion of 250 c.c. of water and a placebo containing no calcium or phosphate ingredients. Subject B has again been chosen as exhibiting a behavior typical of the whole group. As can be seen, the change in ion concentrations is not appreciably different from that of Trial 1.

In contrast to this, the ingestion of 2 Gm. of dicalcium phosphate and vitamin D produced noticeable changes in the ion-concentration trends (Fig. 3). Two observations are immediately apparent. First, the ion concentrations, after an initial period of decrease, start increasing; and second, it is the

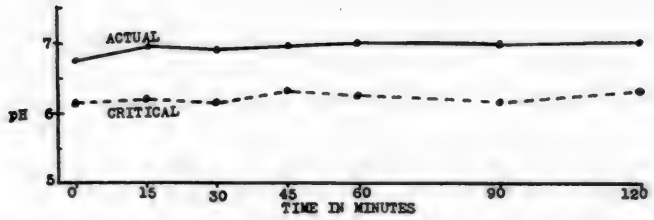

Fig. 5.—Comparison of actual and critical pH values. Part II, Subject B, Trial 1.

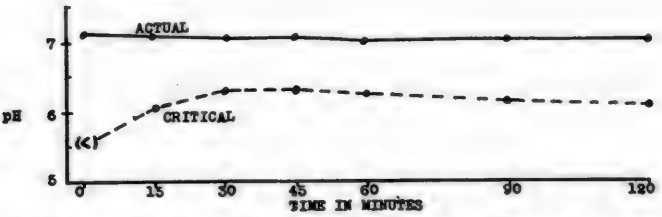

Fig. 6.—Comparison of actual and critical pH values. Part II, Subject B, Trial 2.

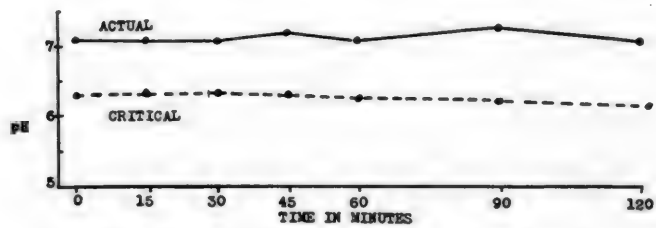

Fig. 7.—Comparison of actual and critical pH values. Part II, Subject B, Trial 3.

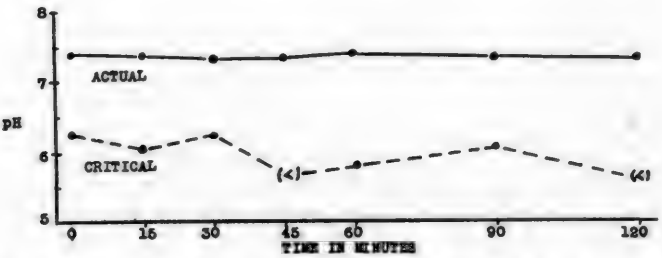

Fig. 8.—Comparison of actual and critical pH values. Part II, Subject F, Trial 3.

phosphate ion which shows the widest variations. Indeed, the calcium-ion fluctuations are within such a narrow range it may be questionable as to whether it increases at all. The behavior of the calcium- and phosphate-ion changes in another individual of the group is shown in Fig. 4. Here, the ion-concentration trends are perhaps better illustrated.

The critical salivary pH values of all the subjects were calculated[1] for the three trials and compared to the actual pH values (Figs. 5, 6, 7). The importance of this comparison lies in the fact that if the critical pH and actual pH differ by more than one pH unit, then the saliva will probably exhibit a definite tendency to form calculus.

Subject B, still typical of the majority, exhibits little evidence of having saliva that would form calculus easily. In contrast to this, a comparison of the salivary critical pH and actual pH values for Subject F (Fig. 8) shows that the values differ by more than one unit and hence his saliva should exhibit an increased tendency to form calculus. Clinical investigation confirms this conclusion.

In this group the correlation of the results of pH value comparisons to the presence of calculus, as determined according to the method outlined by Rapp,[1] was rather high. Seven of the ten subjects were calculus free according to clinical findings. They should have been calculus free according to the salivary indications found. Three of the individuals had calculus. Two of these three subjects had salivary indications which placed them in this category, but the third individual exhibited no salivary indication of a tendency to form calculus.

CONCLUSIONS

1. The intake of water, up to at least 250 c.c., has little to no effect upon the usual changes in salivary concentration of calcium and phosphate ions.

2. The ingestion of a high concentration of $Ca_3(PO_4)_2$ in solution of approximately 250 c.c. results in an increase in the salivary phosphate-ion concentration and may or may not result in an increase of the calcium-ion concentration. These concentration changes in the saliva are relatively unimportant in calculus forming tendency, unless the critical pH of the saliva differs from the actual pH by one or more units. If the difference is more than one pH unit, then the concentration changes increase the tendency of saliva to deposit calculus.

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THE TOOTH PULP AS AN ALGESIMETRY SITE

STANLEY C. HARRIS AND NORMA E. BRANDEL

Department of Physiology and Pharmacology, Northwestern University Dental School, Chicago, Ill.

THIS presentation constitutes a preliminary report of a study still in progress. Sufficient work has been done, however, to enable us to present some interesting and provocative information at this time. The ultimate aim of this research program is to evolve an instrument and a method which will provide reliable and valid information on the efficiency of analgesic drugs. Examination of the existing literature in this area of pharmacology reveals the need for far more precise methods than those now available.

We have selected and used the tooth pulp as the receptive site for stimulation because the consensus indicates that excitation of the tooth pulp is perceived only as a painful sensation. The stimulating agent has been 60-cycle electric current because of its desirable exciting effect on nervous tissue as well as the ease with which it can be controlled and measured.

METHODS

Certain elements of our procedure which have remained uniform are described first. After the human subject is comfortably seated near the algesimeter, a pointed tantalum electrode is placed in a pit of a metal filling. With one hand, the subject holds the long handle of this electrode, keeping the contact placed. His other hand is immersed in a saline bath with the indifferent electrode of the instrument. The operator turns on the instrument and slowly increases the current delivered until the subject feels a brief, repetitive, and slightly painful sensation. When the subject reports his threshold has been reached (by a grunt), the operator opens a switch which breaks the current and then, for the first time, observes the reading of the potentiometer. All machines and methods have been designed so that neither subjects nor operator could know the magnitude of the applied current until threshold has been reported.

The algesimeter was a modification of the one described by Goetzl, Burrill, Ivy, and Harris.[1] In our instrument, the primary coil rotated through a fixed path while the secondary was also in a fixed position. The current was varied from 0 to 250 microamperes by the inclusion in the circuit of a 250 ohm potentiometer, in series with the subject. The dial of the potentiometer was calibrated in centivolts (0.01 v.).

Presented at the Eighty-Ninth Annual Session of the American Dental Association, Chicago, Ill., 1948.

Received for publication, April 18, 1949.

Four control threshold determinations were made at five-minute intervals and seven postmedication determinations were made at fifteen-minute intervals on each of two teeth in each subject.

RESULTS

At first glance the results derived from this experiment might be considered quite satisfactory. Statistical treatment of a series of data obtained from 7 subjects on 6 different days and another 7 subjects on 8 days revealed a very high degree of reliability or uniformity insofar as the control readings were concerned. In all there were 784 observations made, that is, 4 times on 2 teeth in each of 14 subjects on 6 or 8 different occasions. The analysis of variance of these data appears in Table I.

TABLE I
ANALYSIS OF VARIANCE CONTROL THRESHOLDS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO*	PROBABILITY
Subjects	13	15,587	62	<0.01
Teeth	1	20,235	81	<0.01
Days	6	4,224	17	<0.01
Trials	3	13	0.05	>0.05
Discrepance	760	251		

*Mean square of source/mean square of discrepance.

As expected, there were very significant differences between the thresholds of subjects and the thresholds of the two teeth in the same head; probability (P) less than 0.01 in both cases. Not anticipated, but existing nevertheless, was a significant difference between the thresholds of teeth on different days, P less than 0.01. The probability values for the above indicate that there is less than one chance in one hundred that these differences could be due to coincidence. There was no significant difference between the thresholds obtained at five-minute intervals on the same tooth. This is evidence of a uniform threshold within the limits of the experiment as performed.

Let us now examine the results obtained when drugs are given to the fourteen subjects. The drugs were: 1 gr. of codeine phosphate; 5 gr. of aspirin plus 5 gr. of Phenacetin; and calcium lactate, as a placebo. There was a fourth run compared with the effects of these drugs—a run in which no medication was given. To minimize the influence of suggestion, the three drugs were prepared in capsules which all looked alike. Further, the drugs were given to the subjects in different orders and the operator did not know which drug was given on any occasion until the entire cycle of the experiment had been completed. In Fig. 1 are presented the mean responses to these drugs and the "dry" run, expressed as increments from the premedication average threshold. Statistical treatment of these curves, by t ratios, reveals that the only significant differences between effects was between each of the drugs and the "dry" run. There were no significant differences between any of the drugs.

One measure of the reliability of the procedure is to compare the increments of the two teeth in each head, for each category of medication. The results of such comparison are presented in Table II.

TABLE II

CORRELATION OF INCREMENT, TWO TEETH IN SAME HEAD

AGENT	NO. OF PAIRS	CORRELATION COEFFICIENT
Codeine	90	0.70
Placebo	90	0.60
Antipyretic	90	0.45
Nothing	90	0.39

There is less chance than one in one hundred of these correlations being coincidental. It is also interesting to note that the highest degree of correlation occurred with the most potent clinical analgesic agent used, lowest when no medication was given.

Another measure of reliability is the reproducibility of changes due to medication on two different occasions. One grain of codeine was given to seven of the subjects on two different days. The coefficient of correlation between these increments of thresholds was 0.52. There are about five chances in one hundred that this correlation could be coincidental.

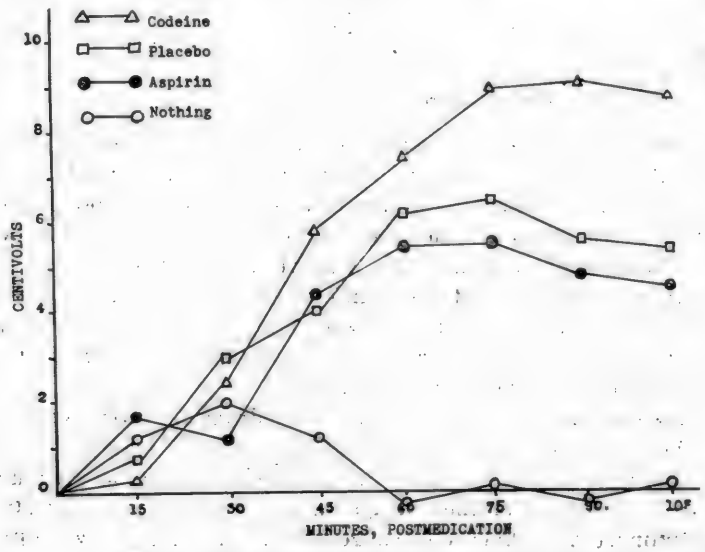

Fig. 1.—Mean increment from premedication threshold.

DISCUSSION

We have found the resting threshold to be of quite different magnitude in different teeth. This does not seem too difficult to understand because the fillings in the teeth are obviously of much different depth and volume. It is presumed that the more hard dental tissue the current must penetrate, the higher the voltage is necessary to deliver enough current to excite the tissues. The realization admits one weakness of the instrument used for this study; we have measured voltage while the amperage was the exciting agent. Inasmuch as the

instrument had only 350 ohms of resistance in the circuit with the subject, changes in the resistance of the circuit could cause alteration in the apparent threshold. To overcome this difficulty, a new machine has been built which has 10,000,000 ohms of resistance in circuit with the subject who has no more than 100,000 ohms, even through an intact tooth.[2]

An explanation for the changes in threshold of a tooth from day to day is not easily made. Since the two teeth in the same head do not always change in the same direction, it is unlikely that changes in the central nervous system are responsible. The most plausible reason seems to be that oxidative or other chemical changes occur on the surface of the restoration.

Although the uniformity between trials taken during the control periods had high significance, it is recognized that this might be due, in part, to relative insensitivity of the instrument, that is, the potentiometer does not break the current into small enough units. Perhaps this is the reason that 1 gr. of codeine does not produce a significantly greater elevation of threshold than does 10 gr. of aspirin.

That aspirin and placeboes produce comparable and significant increments of the tooth pulp threshold is not untenable. Insofar as the placebo effect is concerned, it is not new knowledge that suggestion can alter the reaction or recognition of pain. As a matter of fact, suggestion is a common therapeutic procedure. If one accepts that fact that placeboes can raise the pain threshold, then our data indicate that aspirin is no more analgesic than placeboes. This may well be the case. We do not deny that aspirin compounds have relieved our headaches, but we are inclined to believe that some mechanism, other than analgesia, is involved.

If these experiments have contributed nothing else, they make one point very clear. It is essential in the study of a sensation as subtle as pain that the responsiveness of every subject to a placebo be determined before any effort is made to compare other drugs.

The correlations obtained between the two teeth in the same head are very interesting. Although they are significant statistically, it is desirable to have even higher uniformity of response. One element which we have come to recognize is that the interval between tests may have considerable bearing on uniformity. It was noticed in this series of data that the thresholds were not as uniform when the intervals between tests were 15 minutes instead of five minutes. We are exploiting the influence of recall on the consistency of threshold in a tooth and hope to determine the optimum interval between tests.

That we have obtained the highest degree of correlation in the codeine run and the lowest in the "dry" run may be consistent with the opinion that analgesia is a phenomenon of the highest nervous centers. It would seem that if this is the case, the two teeth would follow the most parallel course when perception of all painful sensation is dulled, while the response would be least parallel when imagination is operating. In our search for highly uniform results, we recognize that parenteral administration of the drugs would probably improve the statistics considerably. This will be done.

Why the correlation between responses on two different exposures to codeine is not higher cannot be answered at this time. It is hoped that improved instruments and methods will improve this type of response.

The fact that all three drugs produced effects (Fig. 1) similar to those described clinically suggests that ours is a valid method. Extension of these studies to include more drugs, different doses of the same drugs, and much larger groups of subjects, will no doubt clarify this point.

SUMMARY

The results of an experiment using the tooth pulp as the receptive site and 60-cycle induced current as a painful stimulus are analyzed and discussed.

The uniformity of premedication thresholds of two teeth in each of fourteen subjects, although high, is recognized as being due perhaps, to insensitivity of the instrument.

The reliability of threshold changes, after oral medication with codeine phosphate, 1 gr.; calcium lactate placeboes; aspirin, 5 gr., and a "dry" run is significant as determined by parallelism of response in pairs of teeth in the same head.

The fact that placeboes cause a significant increment of the tooth threshold indicated the importance of using placebo runs in any comparative study of analgesic drugs.

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HYPOPROTEINEMIA IN RELATION TO THE DENTAL TISSUES

H. A. HUNTER, D.D.S.

Faculty of Dentistry, University of Toronto, Toronto, Ontario

INTRODUCTION

THE role played by proteins in the formation, cellular proliferation, histodifferentiation, and general metabolism of the odontogenic tissues is one that has received relatively little attention in the past. Dental literature contains a wealth of information on the effects of carbohydrates, vitamins, minerals, and many of the endocrine secretions on the teeth and their supporting structures. The delay in the investigation of the function of proteins in this regard was largely due to inability to obtain the various amino acids in chemically pure form. It was not until about 1938 that such materials were available in quantity, thus permitting a critical control of ingested amino acids under experimental conditions.

The very existence of protein material in enamel tissue was questioned until Gies[1] showed, in 1926, that the organic matrix was protein in nature. Following this, Rosebury,[2] in 1930, came to the conclusion that this protein was keratin, but since then Engel[3] has been able to show that, instead, it is a glycoprotein.

All tissues include protein in their physical composition, but in widely varying amounts and types. Therefore, in order that there may be continued optimal growth and replacement of such tissues, it is necessary that the proper amino acids be present in adequate amounts in the diet to act as basic ingredients for the synthesis of new tissues. These amino acids may be utilized either as the basic amino acid itself or by the analysis of proteins present in the diet. Of the twenty-two amino acids known to be present in the various proteins, there are nine which are essential in the rat's diet and one additional one, arginine, which the rat cannot produce in adequate quantity.

Because of the inability of the rat to synthesize these essential amino acids, they must be in the diet. The quantitative need for these individual amino acids varies over a considerable range. Table I gives the levels at which the amino acids were fed in this experiment.

With no precedent as a guide in the direction along which to conduct an investigation in this field, the initial program was established in the following manner.

METHOD

Since caries was not an anticipated feature of the experiment, female albino rats were chosen and were divided into eight groups of four animals each. The experiment consisted of varying the dietary intake of protein in

This report is based on work done at and supported by a Fellowship in the Zoller Memorial Dental Clinic, University of Chicago, Chicago, Ill.

Received for publication, April 1, 1949.

TABLE I
QUANTITATIVE VALUES FOR AMINO ACIDS FED IN THIS EXPERIMENT

	PER CENT
Lysine	0.975
Tryptophane	0.185
Histidine	0.348
Phenylalanine	0.536
Leucine	1.250
Isoleucine	1.340
Threonine	0.804
Methionine	0.360
Valine	1.440
Arginine	0.510
Alanine	0.577
Aspartic acid	0.650
Cystine	0.037
Glutamic acid	2.460
Glycine	0.515
Tyrosine	0.660

the experimental groups and, along with the control groups, observing any macroscopic changes that took place, while at the same time recording the rate of eruption of the lower incisors. Histological preparations were made of the first five groups to further study the tissues in microscopic detail.

Group No. 1 had a diet complete in all known respects except that it lacked one of the essential amino acids—methionine. Group No. 2 was similar, but was lacking in lysine. Group No. 3 was a control for the previous two and it was fed a complete diet, containing the same constituents as the above, but with 9 per cent casein added as the source of protein. Group No. 4 was again the same basic diet, but was lacking in any protein whatsoever. These four groups constituted a unit, and their four diets had the same caloric value of 3.2 calories per gram. Group No. 5 was fed Purina Laboratory Chow gram for gram with the others as an additional control group. Group No. 6 was fed the same diet as Group No. 3 except that it was given ad libitum. Group No. 7 got Chow ad libitum in whole form, whereas Group No. 8 was given Chow ad libitum after it had been ground to powder form.

At the start, the animals were 15 weeks old and weighed between 199 grams and 225 grams, having been raised on Chow after weaning. Those animals in the first five groups were given 15 Gm. of diet a day, equivalent to 48 calories for each rat. Each morning the unconsumed food from the previous day's feeding was weighed back so that a record of the actual food ingested was obtained. Since experience has shown that the animals will not continue to eat 15 Gm. a day of the deficient diets, the consumption in the five groups was kept isocaloric by feeding the same quantity to all groups equal to that of the group consuming the least amount the day before. In this way it was possible to evaluate changes in the tissues which might otherwise be due to the effects of inanition alone. This was the purpose of Group No. 3.

The last three groups were fed ad libitum, but their unused food was also weighed back every day to see how much the animals would eat on their own volition. Groups No. 7 and No. 8 were included to see if there was any stimula-

tion either in the rate of eruption of the incisors or in the animal's appetite because of the masticatory factor necessary to break up the hard pellets of Chow.

Each week the animals were weighed, having been fasted the night before, and every other week the eruption of the lower incisors was measured. The measurements were done by anesthetizing the animals with ether and marking the enamel at the level of the free margin of the gingivae with a knife-edged stone. The distance between these marks and the previous mark nearer the incisal edge was measured along the curvature of the enamel surface and recorded in millimeters.

The experiment was continued for a period of thirteen weeks, at which time the animals in Group No. 4 were so emaciated that it was feared they would shortly die from the effects of malnutrition. All the animals were autopsied, the heads being fixed in 10 per cent formalin and the soft tissues in Zenker's formol. At the same time, serum protein and hemoglobin values were calculated. Sections of the soft tissues were prepared with hematoxylin and eosin and a set of Mallory's connective tissue stained slides were made of the myocardium and skeletal muscle. The left mandible was serially sectioned parallel to the long axis of the incisor and stained with the Larsen-Levin modification of Masson's stain.

The composition of the synthetic diets made up for the first five groups was as shown in Table II.

TABLE II
SYNTHETIC DIETS

	GROUP NO. 1 WITHOUT METHIONINE	GROUP NO. 2 WITHOUT LYSINE	GROUP NO. 3 9% CASEIN	GROUP NO. 4 WITHOUT PROTEIN
Carbohydrate (Dextrin)	58.8 Gm.	58.8 Gm.	61.26 Gm.	71.0 Gm.
Fat (Maxola Oil)	4.0 Gm.	4.0 Gm.	4.0 Gm.	4.0 Gm.
Nitrogen source	12.12 Gm.	12.12 Gm.	10.58 Gm.	–
Fibre (Ruffex)	5.0 Gm.	5.0 Gm.	5.0 Gm.	5.0 Gm.
Salt mixture*	4.0 Gm.	4.0 Gm.	4.0 Gm.	4.0 Gm.
Vitamin solutions†	2.7 c.c.	2.7 c.c.	2.7 c.c.	2.7 c.c.
Water	13.3 c.c.	13.3 c.c.	12.46 c.c.	13.3 c.c.
Total diet	100.0 Gm.	100.0 Gm.	100.0 Gm.	100.0 Gm.

*A variation, by the addition of traces of $CuSO_4$ and $ZnCl_2$, of Hawk and Oser's[4] modification of Osborne and Mendel's salt mixture.

†Vitamins were supplied in the diet in the following amounts: "Mead's" oleum perchomorphum, 0.3 drops; choline chloride, 2.00 c.c.; nicotinamide, 0.284 c.c.; pantothenate Ca, 0.175 c.c.; pyridoxine, 0.064 c.c.; riboflavin, 0.108 c.c.; thiamin, 0.154 c.c.; total 2.7 c.c., where 1 c.c. equals 10 mg. of vitamin.

RESULTS

Table III shows the biweekly rate of eruption of the lower incisors of all animals, measured to the nearest one-fourth millimeter.

From Table III one can see that there is no statistically significant difference or trend in the rate of eruption of the incisors between the various groups. It may be seen that individual animals have a greater or lesser average rate or eruption than some of the others, but this appears to be a constitutional factor

and not dependent on the environmental stimuli as altered in this experiment. The extremes in eruptive rate ranged from 3.00 mm. to 3.81 mm. per week. In spite of the fact that animals in Group No. 4 received no protein for thirteen weeks, their dental tissues continued to proliferate and grow at a normal rate. This must, then, suggest that in order to accomplish this, the odontogenic tissues were obtaining their amino acids from the protein source within the animal's own body and at the expense of some other organ or tissue. This continued growth and eruption at apparently normal rate, took place regardless of caloric intake level as may be seen in Fig. 1 where Groups No. 4 and No. 6 are illustrated for comparison.

TABLE III

	ANIMAL	BIWEEKLY INTERVALS 1	2	3	4	5	6	TOTAL GROWTH	AVERAGE WEEKLY GROWTH	GROUP AVERAGE WEEKLY GROWTH
Group No. 1	1.	6.50	5.75	6.50	6.25	7.75	8.50	41.25	3.44	3.52
	2.	6.00	6.75	6.00	7.75	8.00	8.00	42.50	3.54	
	3.	6.75	7.00	6.00	7.25	7.50	7.50	42.00	3.50	
	4.	7.25	7.25	6.50	7.75	7.50	7.00	43.25	3.60	
Group No. 2	1.	6.25	6.75	6.25	5.50	–	8.50	–	3.33	3.60
	2.	7.00	6.50	6.25	7.50	7.50	7.50	42.25	3.52	
	3.	7.75	8.00	7.00	8.50	7.75	8.00	47.00	3.92	
	4.	6.75	7.25	7.00	7.50	8.25	7.00	43.75	3.64	
Group No. 3	1.	6.50	6.25	5.00	6.25	6.00	6.00	36.00	3.00	3.07
	2.	6.75	5.75	5.75	6.00	6.00	6.00	36.25	3.02	
	3.	7.00	6.75	6.00	6.00	6.00	6.50	38.25	3.19	
	4.	6.75	5.75	6.00	6.50	5.75	6.25	37.00	3.08	
Group No. 4	1.	7.00	6.75	7.00	7.00	6.75	7.50	42.00	3.50	3.36
	2.	6.25	6.50	6.00	7.25	6.50	7.75	40.25	3.35	
	3.	6.25	6.50	6.00	7.00	6.50	6.50	38.75	3.23	
	4.	7.00	6.75	6.50	6.50	6.75	7.00	40.50	3.38	
Group No. 5	1.	6.75	6.25	6.50	6.25	5.75	7.00	38.50	3.21	3.43
	2.	7.25	7.25	7.25	6.50	7.00	7.25	42.50	3.54	
	3.	7.75	6.50	6.50	6.75	6.50	7.75	41.75	3.48	
	4.	7.50	6.75	6.75	7.25	6.50	7.25	42.00	3.50	
Group No. 6	1.	8.00	7.25	7.25	7.75	8.00	6.75	45.00	3.75	3.54
	2.	7.50	7.50	7.25	7.75	7.00	7.50	44.75	3.73	
	3.	7.75	7.25	7.00	6.75	6.50	7.25	42.50	3.54	
	4.	6.25	6.00	6.50	6.00	5.25	6.50	36.50	3.04	
Group No. 7	1.	7.75	7.25	6.75	6.25	6.25	7.50	41.75	3.48	3.69
	2.	8.00	7.75	7.75	7.25	7.25	7.75	45.75	3.81	
	3.	7.75	7.50	7.00	7.25	7.00	8.25	44.75	3.73	
	4.	7.75	7.00	7.50	7.00	6.75	7.75	43.75	3.64	
Group No. 8	1.	7.00	6.50	6.25	6.25	6.75	7.75	40.50	3.38	3.47
	2.	7.25	7.00	6.50	7.25	6.50	6.75	41.25	3.44	
	3.	7.50	6.50	6.25	6.75	7.00	7.00	41.00	3.42	
	4.	7.50	7.75	6.25	7.25	7.25	7.50	43.50	3.63	

Table IV is the same as Table III, except that the biweekly increments are plotted as either an increase over, a decrease from, or the same as that of the previous interval. Column 1 corresponds to Column 2 of Table III.

Examination of the percentile values in the five measured periods shows a rather significant trend in one direction in the first, second, and fifth intervals. Just what these trends portend is a matter for debate; it may be that the environment of the animals (such as temperature, humidity or disturbances of other

TABLE IV

	BIWEEKLY INTERVALS				
	1	2	3	4	5
Group No. 1	–	x	–	x	x
	x	–	–	x	x
	x	–	–	x	x
	0	–	x	–	–
Group No. 2	x	–	–	x	–
	–	–	x	0	0
	x	–	x	–	x
	x	–	x	x	–
Group No. 3	–	–	x	–	0
	–	0	x	0	0
	–	–	0	0	x
	–	x	x	–	x
Group No. 4	–	x	0	–	x
	x	–	x	–	x
	x	–	x	–	0
	–	–	0	x	x
Group No. 5	–	x	–	–	x
	0	0	–	x	x
	–	0	x	–	x
	–	0	x	–	x
Group No. 6	–	0	x	x	–
	0	–	x	–	x
	–	–	–	–	x
	–	x	–	–	x
Group No. 7	–	–	–	0	x
	–	0	–	0	x
	–	–	x	–	x
	–	x	–	–	x
Group No. 8	–	–	0	x	x
	–	–	x	–	x
	–	–	x	x	0
	x	–	x	0	x
Total (x)	8	6	17	10	23
Total (–)	21	20	11	16	4
Total (0)	3	6	4	6	5
or:					
Total (x)	25.0%	18.7%	53.1%	31.2%	71.8%
Total (–)	65.7%	62.5%	34.4%	50.0%	12.5%
Total (0)	9.3%	18.8%	12.5%	18.8%	15.7%

Legend: (x) Any increase over the previous measurement.
(–) Any decrease below the previous measurement.
(0) The same as the previous measurement.

sorts as noise or psychic upsets, etc.), in some way, influences their body processes. Though no solution is offered at present to explain this, nevertheless it is a provocative point.

Table V shows the weekly percentage change in weight of the animals. From it, it may be seen that the protein-free group lost weight most rapidly and that the methionine-free group lost weight almost half as fast again as the lysine-free group. Since three of the four animals in Group No. 3 were a little below their starting weight, the dietary intake was of insufficient caloric value for weight maintainance, but the difference between this group and Groups No. 1, No. 2, and No. 4, which had the same caloric value, is so great that the lack of protein is undoubtedly responsible.

Table V

Percentage Loss or Gain in Weight

	Animal	First week	Second week	Third week	Fourth week	Fifth week	Sixth week	Seventh week	Eighth week	Ninth week	Tenth week	Eleventh week	Twelfth week	Thirteenth week	
Group No. 1	1.	–3.0	–11.0	–15.1	–16.6	–18.6	–21.6	–23.0	–26.6	–30.0	–32.2	–34.7	–36.7	–37.7	
	2.	–4.8	–11.9	–16.3	–19.1	–22.0	–26.3	–32.5	–32.5	–33.5	–36.7	–38.3	–39.7	–40.0	
	3.	–0.9	–12.2	–17.4	–17.8	–21.0	–22.0	–24.9	–24.4	–26.8	–27.7	–30.0	–31.0	–32.4	
	4.	–5.4	– 9.9	–14.9	–17.1	–18.9	–22.5	–23.8	–25.2	–28.8	–30.6	–34.2	–35.6	–35.6	
Average		–3.5	–11.3	–15.9	–17.6	–20.1	–23.0	–26.1	–27.2	–29.8	–31.8	–34.3	–35.8	–36.4	Loss
Group No. 2	1.	–1.8	– 8.1	– 6.3	– 8.1	–12.2	–14.4	–14.4	–14.9	–18.5	–19.4	–21.6	–21.6	–23.0	
	2.	–2.0	– 8.0	– 8.5	– 9.5	–13.0	–15.6	–17.6	–16.6	–18.6	–18.6	–20.6	–23.1	–23.2	
	3.	–0.9	– 8.0	– 9.4	–10.3	–13.1	–15.0	–15.0	–16.0	–20.0	–19.7	–21.1	–23.0	–24.0	
	4.	–1.9	– 6.8	–11.2	–11.2	–15.5	–17.5	–18.0	–17.5	–22.8	–23.3	–27.2	–28.6	–29.2	
Average		–1.7	– 7.7	– 8.9	– 9.8	–13.4	–15.6	–16.5	–16.5	–20.0	–20.3	–22.6	–24.1	–25.0	Loss
Group No. 3	1.	3.7	5.0	8.7	10.1	11.0	8.3	8.7	9.6	6.9	8.3	6.4	3.7	1.8	
	2.	7.2	9.7	10.1	12.6	16.4	15.9	16.4	18.4	15.9	16.4	14.0	10.1	12.1	
	3.	5.1	3.0	4.0	7.6	8.1	9.1	5.6	12.6	10.1	7.1	3.0	– 1.5	– 3.5	
	4.	4.2	7.9	7.0	8.4	9.8	10.7	14.9	9.3	9.3	7.0	3.0	– 0.5	– 1.4	
Average		5.1	6.4	7.5	9.7	11.3	11.0	11.4	12.5	10.6	9.7	6.7	2.9	2.3	Gain
Group No. 4	1.	–4.7	–11.3	–13.6	–16.9	–21.6	–23.0	–25.8	–29.0	–31.4	–32.9	–38.0	–37.1	–39.4	
	2.	–2.3	–11.4	–14.6	–17.3	–22.0	–26.0	–29.2	–31.0	–34.7	–35.6	–37.9	–39.7	–41.5	
	3.	–5.9	–11.2	–13.7	–15.1	–18.5	–23.4	–25.4	–27.8	–35.6	–36.9	–36.6	–44.9	–46.8	
	4.	–3.0	–12.1	–14.1	–17.1	–21.1	–24.1	–27.1	–30.1	–33.1	–35.7	–38.7	–40.7	–42.7	
Average		–4.0	–11.6	–14.0	–16.6	–20.8	–24.1	–26.9	–29.5	–33.7	–35.3	–37.8	–40.6	–42.6	Loss
Group No. 5	1.	3.5	5.5	3.0	6.0	2.5	4.0	4.5	6.0	3.5	2.0	0.5	0.0	4.5	
	2.	5.8	5.3	3.1	3.6	1.8	2.7	1.8	2.2	– 2.7	– 5.3	– 9.8	–11.1	–12.9	
	3.	3.4	2.9	1.0	1.5	2.9	1.5	1.0	1.5	0.0	– 2.4	– 4.4	– 6.3	– 5.3	
	4.	9.3	7.9	8.3	4.6	5.1	1.4	1.9	3.7	– 1.0	– 1.4	– 3.7	– 6.0	– 3.7	
Average		5.5	5.4	3.9	3.9	3.1	2.4	2.3	3.4	– 0.1	– 1.8	– 4.6	– 5.9	– 4.4	Loss
Group No. 6	1.	7.8	8.7	10.7	13.1	16.0	16.0	15.0	16.5	16.0	21.8	25.4	22.3	24.8	
	2.	10.8	11.7	11.7	16.0	14.5	18.3	18.3	20.7	22.5	25.8	23.9	25.4	26.2	
	3.	7.9	8.4	7.4	10.4	11.4	12.4	12.4	23.8	24.3	25.8	29.2	32.2	27.7	
	4.	7.3	10.5	9.1	14.2	15.1	14.2	19.2	18.7	22.8	23.3	23.8	26.9	26.0	
Average		8.5	9.8	9.7	13.6	14.3	15.2	16.2	19.9	21.4	24.2	25.6	26.7	26.2	Gain
Group No. 7	1.	2.5	2.0	3.0	7.9	7.4	6.4	9.9	9.4	11.8	13.8	13.8	16.3	17.7	
	2.	8.3	6.3	10.2	15.1	14.2	13.2	18.1	17.6	19.0	19.0	21.0	22.4	24.4	
	3.	1.9	2.9	4.3	8.1	9.1	8.6	12.9	12.4	10.0	11.0	12.9	14.8	15.2	
	4.	3.6	4.5	5.0	10.0	8.6	10.5	12.3	12.3	10.0	12.7	11.4	15.5	17.7	
Average		4.1	3.9	5.6	10.5	9.8	9.7	13.3	12.9	12.7	14.1	14.8	17.3	18.8	Gain
Group No. 8	1.	6.2	5.8	6.2	13.0	13.0	12.5	20.0	21.0	18.6	19.7	18.8	20.2	20.7	
	2.	7.9	6.9	9.9	12.8	13.8	17.7	18.7	21.7	19.2	16.8	16.3	18.2	20.2	
	3.	3.3	1.4	3.3	7.4	6.5	9.3	9.8	12.1	9.8	12.6	13.5	13.5	15.8	
	4.	0.5	1.5	6.9	10.8	8.8	11.3	15.2	15.7	16.1	19.1	19.1	20.1	22.6	
Average		4.5	3.9	6.6	11.0	10.5	12.7	13.4	17.6	15.9	17.1	16.9	18.0	19.8	Gain

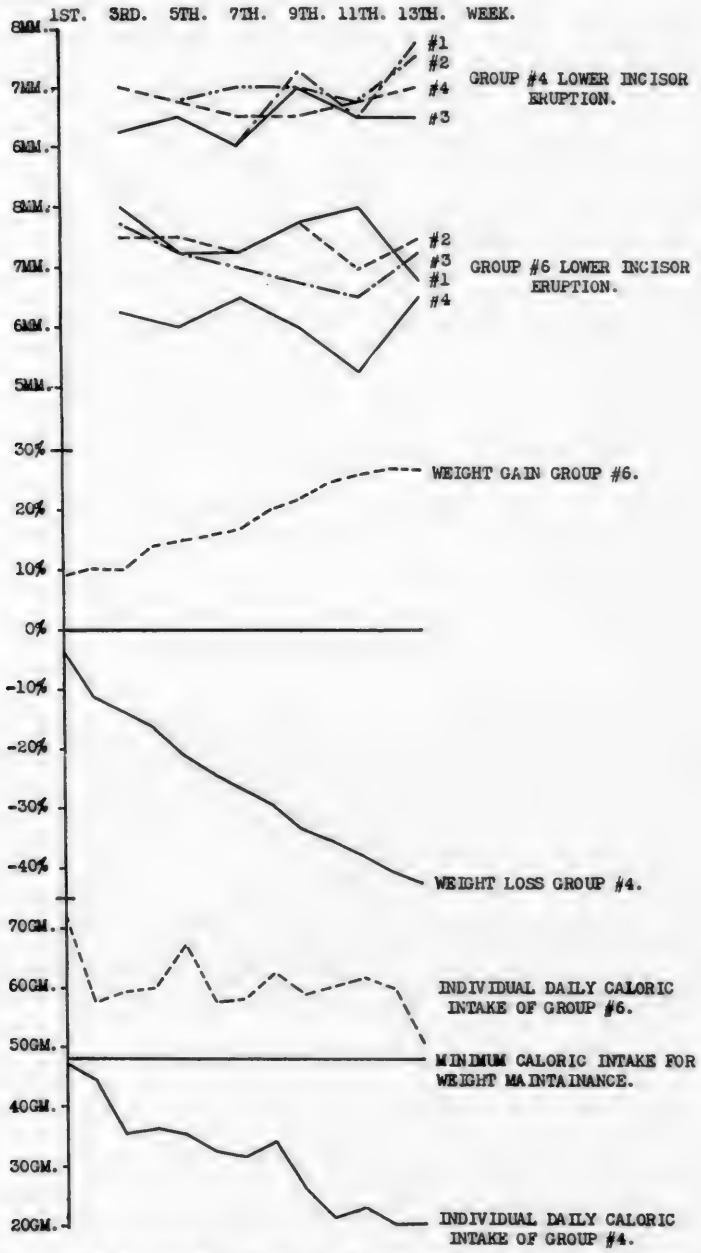

Fig. 1.—Diagram of Groups No. 4 and No. 6 comparing rate of incisor eruption with change in body weight and caloric intake.

The outward changes observed in the animals were: First, the obvious loss of weight of the depleted ones. The three groups lacking in protein, that is, Groups No. 1, No. 2, and No. 4, all showed alopecia, Group No. 2 showing it first, two weeks after the start, and Groups No. 1 and No. 4 showing it ten days later. The coats of these animals also lost their smooth texture and became rough and thinner. The extreme degree of emaciation can best be appreciated by comparing Figs. 2 and 3, taken just before the end of the experiment. The depleted animal (Fig. 2) is No. 3 from Group No. 4 and it has lost 46 per cent of its starting weight. The other is No. 3 from Group No. 6, a control.

During the bi-weekly marking of the lower incisor, a superficial cheek of the oral cavity revealed no evidence of any changes. The incisors themselves, besides their regular eruption, showed a normal curvature and occlusion with their opponents in the maxillae. The normal yellowish-brown pigmentation of the enamel showed no untoward change or variation in the eight groups. The hardness of the enamel appeared to be unchanged insofar as one could tell while marking them with the knife-edged stone. There was no gross evidence of inflammation or ulceration in the oral cavity. After fixation of the heads and excision of the mandible, the molars were examined with the aid of a sharpened explorer and hand magnifying glass for occlusal caries and none was found, though the degree of occlusal wear on the molars varied in individual animals. Two animals were observed to have bits of solder embedded in the occlusal fissures of a molar because of their habit of chewing the wire of their cages.

The right lower incisor and upper right first molar were dissected out of the animals in Groups No. 4 and No. 6. They were cleaned of all adherent tissue debris and then dried in an oven for 48 hours at a temperature of 120 degrees Centigrade. After that time they were weighed twice and the average of the two weighings recorded in Table VI.

TABLE VI

RIGHT LOWER INCISOR			RIGHT UPPER FIRST MOLAR		
Group No. 4	1.	.0614	Group No. 4	1.	.0179
	2.	.0646		2.	.0165
	3.	.0615		3.	.0158
	4.	.0655		4.	.0175
Average		.0634 Gm.	Average		.0169 Gm.
Group No. 6	1.	.0799	Group No. 6	1.	.0177
	2.	.0872		2.	.0158
	3.	.0843		3.	.0162
	4.	.0936		4.	.0185
Average		.0863 Gm.	Average		.0171 Gm.

From Table VI one can see that the incisors of the protein-free group are 26.53 per cent lighter than those of the control group, and that the molars are only 1.17 per cent lighter. This is as would be expected because the molars were formed before the experiment started and thus had little growth requirement. The incisors are composed of tissues, all of which were formed in a depleted environment and thus offers a ready explanation of the reduced weight.

Further measurements were made on the lower incisors. The buccolingual diameter was measured at six equidistant points along its length and an average diameter obtained (Table VII).

TABLE VII
BUCCOLINGUAL DIAMETER

GROUP NO. 4				GROUP NO. 6			
NO. 1	NO. 2	NO. 3	NO. 4	NO. 1	NO. 2	NO. 3	NO. 4
1.19	1.21	1.25	1.22	1.33	1.35	1.34	1.41
1.21	1.22	1.21	1.23	1.35	1.39	1.36	1.46
1.22	1.23	1.26	1.24	1.37	1.39	1.39	1.48
1.16	1.19	1.19	1.16	1.33	1.36	1.36	1.46
1.15	1.15	–	1.13	1.25	1.26	1.23	1.44
–	1.07	1.05	1.11	–	1.20	1.21	1.33
1.19	1.18	1.19	1.18	1.33	1.33	1.32	1.43
Group average 1.18 mm.				Group average 1.35 mm.			

By comparison, the protein-free group has lost 12.41 per cent of this dimension. The only other observation was the lack of adipose tissue and atrophy of the skeletal muscles. The heads were cut off and halved along the sagittal plane and x-rayed along with the dissected-out mandible of the left side. Comparison of these roentgenograms showed no significant difference in the groups in respect to density of the hard tissues, position or shape of the incisors. The anteroposterior length of the skull, measured from the tip of the alveolar process in the premaxilla to the tip of the external occipital crest, showed the depleted groups to be from one-half to one millimeter shorter than the well-nourished animals. Apart from this the over-all appearance of the depleted heads and their dental tissues is surprisingly normal.

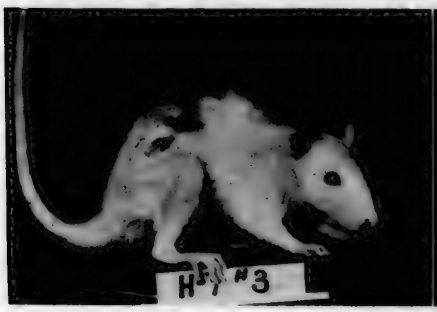

Fig. 2.

Fig. 3.

Fig. 2.—Group No. 4 animal at the end of thirteen weeks' protein-free period. Note extreme emaciation, alopecia, and nonhealing ulcer of the skin.
Fig. 3.—Group No. 6 animal fed a 9 per cent casein diet. A normal control animal.

Table VIII gives the values of serum protein and hemoglobin obtained at time of autopsy. The normal values for serum protein is between 6 and 7 Gm. per cent and for hemoglobin it is 15.6 Gm. per cent (100 per cent in the Sahli method). The results obtained are what might be expected, the figures in Group No. 4 show the lowest values because of its complete lack of dietary protein. The low caloric groups, No. 3 and No. 5, both show normal serum protein levels and Group No. 3 also was a bit low in its hemoglobin concentration.

TABLE VIII

	ANIMAL	SERUM PROTEIN (GM. %)	HEMOGLOBIN (GM. %)
Group No. 1	1.	4.92	13.9
	2.	4.78	14.1
	3.	5.41	14.3
	4.	4.08	13.8
	Average	4.80	14.0
Group No. 2	1.	5.58	13.3
	2.	5.30	13.7
	3.	5.23	13.8
	4.	5.34	13.4
	Average	5.36	13.6
Group No. 3	1.	6.14	14.4
	2.	6.07	13.9
	3.	6.62	15.4
	4.	6.10	14.6
	Average	6.23	14.6
Group No. 4	1.	4.05	10.6
	2.	4.78	10.9
	3.	4.26	8.7
	4.	4.22	12.1
	Average	4.33	10.6
Group No. 5	1.	5.89	15.4
	2.	6.42	16.3
	3.	6.28	16.4
	4.	6.24	16.1
	Average	6.21	16.1
Group No. 6	1.	6.69	15.0
	2.	6.59	15.1
	3.	6.21	14.7
	4.	6.55	14.7
	Average	6.51	14.9
Group No. 7	1.	5.72	14.9
	2.	6.45	15.8
	3.	6.24	15.4
	4.	6.59	15.6
	Average	6.25	15.4
Group No. 8	1.	6.76	16.0
	2.	6.14	16.6
	3.	6.69	16.2
	4.	6.59	16.4
	Average	6.56	16.3

MICROSCOPIC OBSERVATIONS

The principal feature seen in many of the organs and tissues of the body in the depleted groups was atrophy. In the skeletal muscles the fibers are definitely smaller in diameter, but there appears to be no real degeneration, though in a few places there can be seen groups of muscle cell nuclei in serial arrangement suggestive of amitotic division and claimed to be a form of degeneration (Figs. 4 and 5). Both skeletal and cardiac muscle show an increased number of connective tissue cell nuclei per field, but this may only be a relative increase due to the atrophy of the myofibrils. It remains to be demonstrated that there is an absolute increase of connective tissue in such situations.

The livers of the depleted animals showed a remarkably regular architecture. The lobuli are well marked and show no sign of degeneration. The hepatic cords are slightly smaller than normal and the cells show some definite

small droplet vacuolization, but without any pigment. Specific fat stains were not done and so the vacuolization is of debatable significance as it may represent a metabolic phase of the hepatic cells. There was no round cell infiltration about the portal triads.

Fig. 4

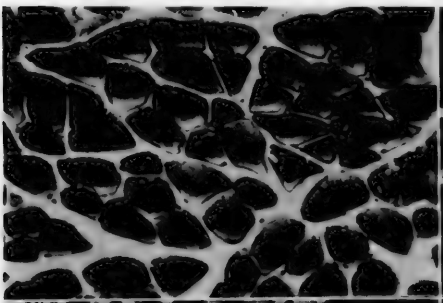

Fig. 5

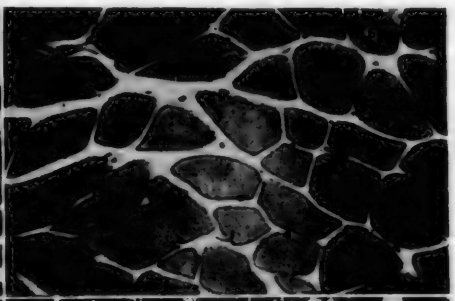

Fig. 6

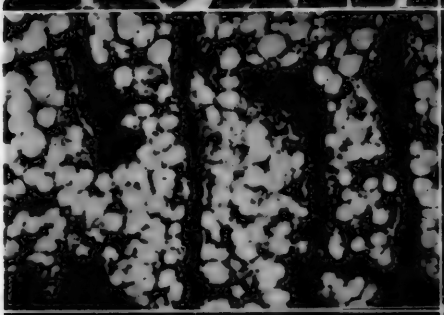

Fig. 7

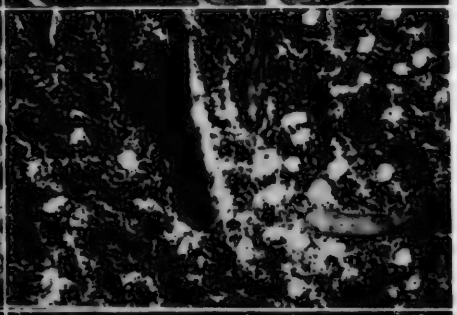

Fig. 8

Fig. 9

Fig. 4.—Sternohyoid muscle of protein-free animal. Note atrophy of the muscle fibers.

Fig. 5.—Normal skeletal muscle from control animal in Group No. 6.

Fig. 6.—Fatty sternal marrow of protein-free animal. There is also some evidence of slight thinning of the trabeculae.

Fig. 7.—Normal sternal marrow.

Fig. 8.—Periodontal membrane of lower incisor of protein-free animal. There appears to be good quantity and distribution of the principal fibers and their attachment to the bone and cementum. It does not appear to differ from the control animal.

Fig. 9.—Periodontal membrane from control animal in Group No. 6 taken from the same relative position along the incisor.

The spleens for the most part showed smaller nodules and the lymphatic sheaths about the arteries were reduced. The lymphatic centers show only occasional mitoses and the number of lymphocytes around the centers are less than in the normal animals. The appearance is that of subnormal lymphopoiesis.

The kidneys appeared to be normal in appearance, with the exception that Animal No. 3 in Group No. 2 showed some indications of a slight pyelonephritis. The perirenal fat is typical of wasting adipose tissue; the nuclei are in a more central position in the cells and there are two to three smaller globules of fat in the cytoplasm.

Most of the adrenal glands of the depleted animals showed a thickening of the zona glomerulosa and in some, the whole cortex seemed less wide than normal. The capsule is thicker too, but may be due to the atrophy of the organ as a whole.

Animal No. 3 in Group No. 4 was the only one to show any ulceration of the gastric mucosa. In the submucosa of many of the animals there was edema and a lesser number showed acute inflammation as well. All the layers of the stomach exhibited some atrophy ranging from the mucous membrane through the muscularis mucosa. Most of the depleted stomachs also showed a deepening of the gastric foveolae.

In the large intestine there is atrophy of the mucous membrane and edema in the submucosa and, though lymphatic tissue is present, it is scarce. The chief feature is a marked reduction in the number of goblet cells.

The ovaries of all the depleted animals showed a marked degree of atrophy. The germinal epithelium was intact and a few primary follicles were present. There were also degenerating follicles, but no intermediary stages between these two, indicating that growth and maturation had ceased.

Examination of the sternal marrow showed quite a variation (Figs. 6 and 7). On the basis of percentage cellularity, the protein-free group, No. 4, ranged from 5 to 10 per cent; the methionine-free group, No. 1, from 5 to 15 per cent; the lysine-free group, No. 2, from 10 to 25 per cent. Group No. 3 was 20 to 40 per cent and Group No. 8 was 30 to 70 per cent cellular. The depleted groups showed a thinning of the cortex and also of the trabeculae with some evidence of osteoporosis. The degeneration of the marrow is not the usual gelatinous or atrophic type as seen in emaciated animals. It is more of a change from a hemopoietic to a fatty marrow.

The microscopic appearance of the lower incisor showed nothing that could be construed as beyond the normal histological limits. The arrangement of the odontogenic tissues is normal in amount, position, and activity. At the apex of the incisor may be seen good proliferation of the enamel epithelium. There is regular differentiation of the inner enamel epithelium into ameloblasts and they in turn produce normal enamel matrix. The odontoblasts and dentin are normal, as is the amount of predentin. The periodontal membrane shows a normal arrangement and distribution of principal fibers (Figs. 8 and 9).

In the interdental papillae between the molars of some of the depleted animals there were lesions of a traumatic nature. Where these occur, one can see sharp, slender, and straight fibers piercing the gingivae between the teeth

and severing the periodontal fibers as they go downward (Figs. 10, 11, and 12). In some sections they can be seen extending down to and impinging upon the alveolar crest bone. More than one fiber was seen to be present in certain lesions surrounded by foreign body giant cells and, near the surface, there were polymorphonuclear leukocytes and necrotic tissue. These lesions were observed in Animals No. 1 of Group No. 1, No. 1 of Group No. 2, and No. 1 and No. 3 of Group No. 4, but none were seen in any of the other groups.

The tongues of both the protein-free and control groups showed no atrophy of the mucosa and both appeared to have the same degree of keratinization of the surface epithelium. Likewise, there was no alteration in the filiform papillae either in form or number. The picture presented was essentially that of a normal tongue in both groups.

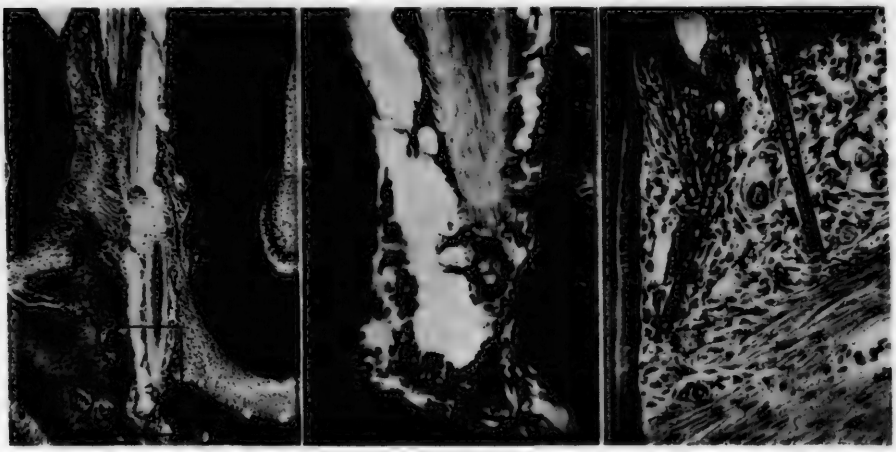

Fig. 10. Fig. 11. Fig. 12.

Fig. 10.—Area between first and second molars of the mandible of protein-free animal. Note the sharp sliver driven into the periodontal membrane severing its attachment and impinging on the alveolar bone.

Fig. 11.—High power of area in Fig. 10. Note foreign body giant cell and leukocytes reacting to the injury.

Fig. 12.—Small silver-like fibers in the gingivae below the epithelial attachment.

DISCUSSION

In the foregoing experiment it has been shown that the groups of animals fed deficient diets, in one case totally free from protein and in two other cases lacking but a single essential amino acid, are not only unable to maintain their body weight, but actually lose weight. The rate at which they do this is different, depending on the specific amino acid that is lacking. The totally protein-free group lost weight most rapidly. All deficient groups showed generalized atrophy of the tissues, but certain organ systems were affected much more so than others. The skeletal musculature and reproductive systems were the most atrophic, though the myocardium was nearly as badly affected. The bone marrow was markedly changed, tending more towards a fatty type of marrow. The gastrointestinal tract was next in order of severity of change.

The urinary system showed no significant atrophic or degenerative changes as seen by their histologic appearance. Urinalyses were not done, so that no evaluation of urinary function can be expressed.

These gross and histological observations along with the low serum protein and hemoglobin values are not surprising when one considers that these animals were in negative nitrogen balance for thirteen weeks. In order to maintain the vital processes in functional order for as long as possible, the less important body tissues were being drawn upon to supply the essential amino acids for the synthesis of the most important organ systems. In this light, it is a matter of some interest to see that the dental tissues appear to possess a high priority on the dwindling supply of essential amino acids. The association of the previously mentioned normal histodifferentiation of the odontogenic tissues along with the measured reduction in weight and diameter of the incisors can be explained on the premise that when such differentiation occurs, the process is perfect and complete, the quality being maintained at the expense of quantity in an environment where insufficient materials are available.

If we accept the hypothesis of a priority system in allocating the available supplies of amino acids, the incisor teeth must rate very high on the list of essential organ systems in order to be able to show such a relatively normal growth and eruptive rate.

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THE SILVER IMPREGNATION METHOD APPLIED TO THE DENTIN MATRIX OF RACHITIC RATS AND TO THAT OF RATS WITH HEALING RICKETS

J. T. IRVING*

Department of Physiology and Pharmacology, Medical School, University of Cape Town, Cape Town, South Africa

THE silver impregnation technic has proved very useful in the interpretation of changes in dental tissues. Orban, Sicher, and Weinmann[8] were the first to apply this method to a study of the hard tissues in their analysis of enamel formation. Subsequently Irving and Weinmann[7] used silver staining to analyze the changes of developing dentin. In the normal tooth, the argyrophil Korff's fibers swell and form a homogeneous matrix of predentin which stains uniformly and deeply (young or "early" predentin). This is a narrow stratum which subsequently differentiates into fibrils and cementing substance (old or "late" predentin); the old predentin appears lighter in color. Here the sharply staining fibrils are seen embedded in the lightly staining cementing substance. When calcification occurs and dentin is formed, the organic cementing substance which holds the lime salts now stains very poorly with silver though well with hematoxylin. This change in the staining reaction has been explained as due to a denaturation of the proteins of the cementing substance during calcification (Weinmann and Sicher[9]). The fibrils stain as well as in the old predentin, but owing to the poor staining of the cementing substance, dentin as a whole takes up the silver stain less well than predentin. In general, tissue staining well with hematoxylin takes up silver poorly and vice versa.

It appeared to be of interest to see how the above interpretation applied to teeth abnormally calcified or in which healing processes were produced by various means. The present paper reports the findings from such teeth, the material used being from observations already published.[2-5]

EXPERIMENTAL

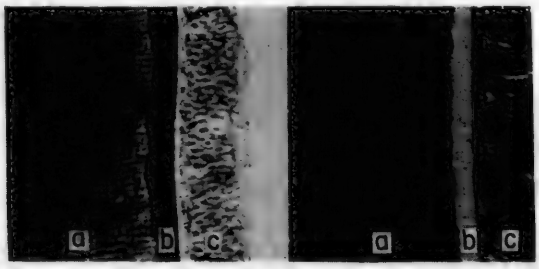
Two groups of young rats of the Wistar Institute strain were studied. The first group were put on to the Steenbock and Black rachitogenic diet No. 2965[10] for 28 days. The second group were fed on a modified diet with a low Ca and a high P content for 28 days. Some rats of both groups were examined at this stage when they had severe rickets; others were killed at various times after healing of rickets had been induced. Healing was initiated in rats of the first group by dosage with vitamin D, starvation, or the addition of P to the diet, and in the second group by vitamin D or the addition of Ca to the diet. Central longitudinal sections of the upper incisor teeth were stained with hematoxylin and eosin (H and E) or by the Gömöri silver impregnation technic.[1]

Received for publication, January 17, 1949.

*Fellow of the University of Cape Town.

RESULTS

The appearance of the normal tooth is shown in Fig. 1. The usual appearance of lightly staining predentin and darkly staining dentin is seen in the section stained with H and E. With the silver stain, the predentin is seen to be in two layers, a narrow zone nearest to the odontoblasts staining more deeply, the young predentin, and that nearer the dentin, staining less deeply, the old predentin. Running through the old predentin will be seen a fine argyrophil line. The writer has noticed this in many sections from normal teeth. It appears to be due to a periodic alteration in the degree of differentiation of the matrix, these fine lines persisting throughout the whole thickness of the dentin. The calcified dentin stains less well owing to the change in the cementing substance, but the fibrils and the walls of the dentin tubules stain very clearly. It will be seen that in general the H and E and silver stains are complementary in their staining appearances.



The microphotographs are all of the mid-region of the labial side of the upper incisor tooth. Original magnification ×240. The sections on the right are stained with hematoxylin and eosin, those on the left with silver impregnation.

Fig. 1.—Rat 1043. Normal appearance. *a* = dentin, *b* = predentin, and *c* = odontoblasts.

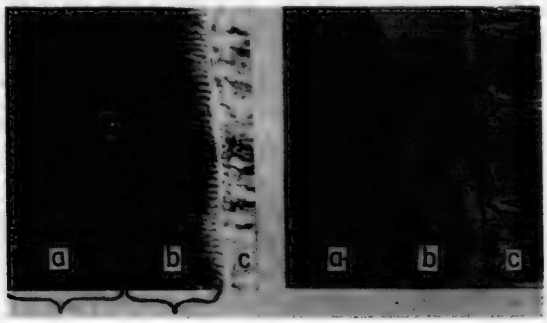


Fig. 2.—Rat 1044, with low P rickets. *a* = dentin, *b* = predentin, and *c* = odontoblasts.

Group I. (Low P rickets)

Untreated rachitic animals.—Fig. 2 is from an animal which was on the Steenbock and Black diet for 28 days. The H and E section shows the wide predentin and the irregular predentin-dentin junction with calcospherites. With the silver stain, the wide argyrophil predentin is seen, the calcified dentin being less avid of the silver stain. In the silver section the distinc-

tion between old and young predentin is not seen, though visible in the H and E section. From the appearance of this and other sections, it would appear that the differentiation of the matrix into fibrils and cementing substance has taken place in the predentin, but that calcification of the cementing substance is delayed. Wih the silver stain a very fine argyrophobe line is visible at the predentin-dentin junction. A similar fine argyrophobe line can sometimes be seen in normal teeth on the boundary of predentin and dentin and is visible in many of the other illustrations. Since the various healing agencies had no effect on the intensity and width of this line, the writer considers it to be associated with the process of the onset of calcification, the dentin fibrils possibly not taking up the silver stain quite so avidly at this point.

Rachitic animals treated with vitamin D.—Fig. 3 shows the appearance of the tooth of a rachitic rat which had been given 18.4 i.u. of vitamin D 7 days before being killed. In the H and E section calcification is seen to have recommenced in the predentin formed after the vitamin was given, as has been already reported by Irving,[2, 3] and no calcification appears to have occurred in the predentin existing before vitamin dosage. The silver stain section, from a

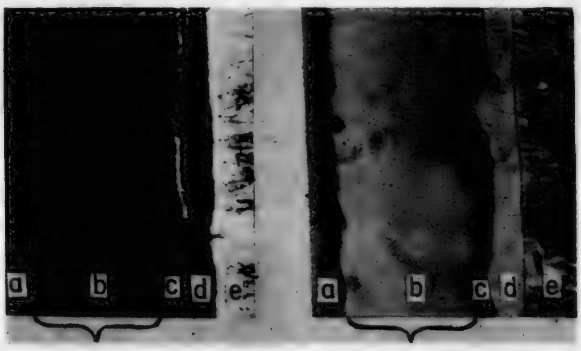


Fig. 3.—Rat 380, with low P rickets, given 18.4 i.u. of vitamin D 7 days previously. *a* = dentin, *b* = pre-experimental predentin, *c* = hypercalcified matrix, *d* = postexperimental predentin, and *e* = odontoblasts.

comparable area, shows the very intense calcification caused by the vitamin, as indicated by a narrow band of very argyrophobe tissue. Two increments of calcification appear to have been laid down. The fine argyrophobe line at the border of the dentin is again seen. Here it is somewhat clearer than in Fig. 2. No change is seen in the rest of the predentin.

In Fig. 4 is seen a later stage of the process of healing brought about by vitamin D (9.2 i.u. given 10 days before death). About 6 incremental layers of newly calcified dentin have been laid down. The silver stain shows the very strongly calcified layer of new dentin. It is surprising that the administration of such small doses of vitamin D should cause such an intense response. It would appear that during the differentiation of the matrix under the action of the vitamin, the formation of dentin fibrils is suppressed almost completely,

so that in a given volume of matrix an excess of cementing substance is present, the process appearing to have a daily cycle, as the hypercalcified dentin is laid down in increments.

The presence of intermediate dentin, which as has been reported by Weinmann and Schour[11] is also laid down in response to vitamin D, is well shown in the H and E section. The same narrow argyrophobe line as that seen in other figures, occurs at the dentin-predentin junction of the silver stained section. In spite of the fact that the vitamin has acted much longer on this tooth, as shown by the greater width of the hypercalcified zone, the line is no wider than in Fig. 3, and thus appears to have nothing to do with the action of the vitamin. The line is not as wide as the layer of intermediate dentin, which has calcified but slightly, and does not seem to be associated with it.

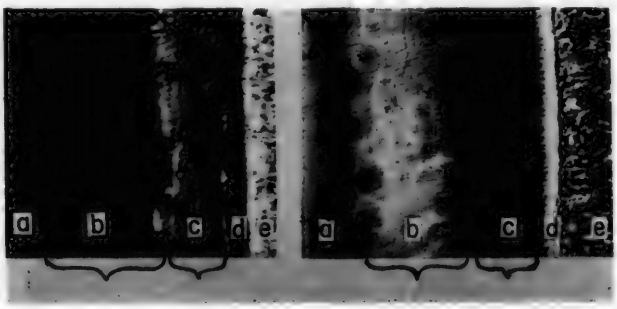


Fig. 4.—Rat 24, with low P rickets, given 9.2 i.u. of vitamin D 10 days previously. *a* = dentin, *b* = pre-experimental predentin, *c* = hypercalcified matrix, *d* = postexperimental predentin, and *e* = odontoblasts.

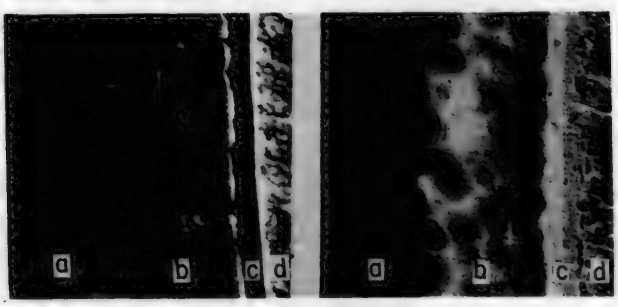


Fig. 5.—Rat 1079, with low P rickets. This rat had its dietary intake restricted for 6 days before being killed. *a* = dentin, *b* = matrix with calcospherites and a hypercalcified layer laid down in response to starvation, *c* = postexperimental predentin, and *d* = odontoblasts.

Rachitic animals with their dietary intake restricted.—Fig. 5 is from a rachitic animal which had its dietary intake restricted for 6 days before being examined. This treatment causes an indiscriminate formation of colcospherities in the existing predentin right up to the edge of the calcified dentin.[5] Later the calcification process is more concentrated near the odontoblasts where predentin of normal width develops. This is well seen in the H and E

section. The silver stain shows that calcification has occurred as calcospherites throughout the predentin. A narrow strip of hypercalcified tissue is seen at normal predentin width from the odontoblasts. As in the other teeth, the narrow argyrophobe line is seen on the edge of the dentin in the silver section. A comparison of Figs. 3 and 5 shows how differently starvation and vitamin D affect the tooth during the healing process.

Rachitic animals given extra P in the diet.—This causes exactly the same response in the predentin as does starvation.[4] The H and E and silver sections thus had an appearance identical with that of Fig. 5.

Group II (Low Ca rickets)

Untreated rachitic animals.—The appearance of the H and E and silver stained sections were in general the same as those already reported for the rats with low P rickets.

Rachitic animals given vitamin D or extra Ca in the diet.—Both these treatments cause an indiscriminate laying down of calcospherites in the existing predentin, and later a hypercalcification of new predentin formed at normal width from the odontoblasts (Irving[3, 4]). The appearances of the H and E and silver sections were the same as those caused by starvation of rats with low P rickets, as seen in Fig. 5.

COMMENT

The action of healing agents on rachitic dentin appears to be at least twofold. During rickets, the dentin matrix differentiates, but the final process of denaturation and calcification of the cementing substance is postponed. The action of healing agents is to cause the final processes of calcification to occur sooner, the exact position of the calcification depending on the healing agent used. As healing becomes complete, the agent causes the denaturation and calcification of the cementing substance to take place 24 hours after the matrix is laid down.

Hematoxylin and silver appear to be complementary in their staining of rachitic dental material, as they are with dental tissues treated in other ways (e.g. Sr[7], F[6]). As pointed out by Weinmann and Sicher[9] and Irving and Weinmann,[7] it can be stated quite generally that highly basophilic structures are argyrophobe, not only in dentin, but also in enamel and bone. Further, the silver stain appears to reveal details not brought out by H and E staining. This can be well seen in comparing the various figures, for example, the fine argyrophobe line bounding the edge of dentin which is not obvious with H and E staining. In addition the contrast in the detail of the dentin matrix is more clearly seen with silver. The writer does not feel that the silver stain should replace the standard H and E method, which is the most generally useful stain for teeth, but he would suggest that silver impregnation be employed more widely in conjunction with H and E, since certain interpretations can only be made by its use.

SUMMARY

1. The incisor teeth of rachitic rats, and those of rachitic animals in which healing had been induced by various procedures, have been stained with

hematoxylin and eosin and by the method of silver impregnation, and the appearances compared.

2. As has been reported for other tissues and for teeth from rats treated in other ways, so here also basophilic tissues were found to be argyrophobe, and vice versa.

3. In both low Ca and low P rickets, differentiation of the dentin matrix into fibrils and cementing substance takes place, but calcification is delayed.

4. Agents causing calcification to recommence in the teeth of rachitic rats have at least two actions: they cause calcification of the cementing substance to be accelerated and finally to occur in the normal situation; and they also affect the differentiation of new matrix in such a way that more cementing substance and fewer fibrils are laid down.

The expenses of this work were defrayed by grants from the Staff Research Fund, University of Cape Town, and the South African Council for Scientific and Industrial Research.

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DISTRIBUTION OF RADIOACTIVE GALLIUM IN THE TEETH AND JAWS OF EXPERIMENTAL ANIMALS

JAMES A. ENGLISH, B.S., D.D.S., M.S., AND H. C. DUDLEY, Ph.D.

Naval Medical Research Institute, Bethesda, Md.

INTRODUCTION

SINCE the discovery by Becquerel (1896) that radiations from uranium ore will effect a photographic emulsion,[1] many radioactive elements have been studied by refined technics of his original method.[2, 3] The first extensive application of this phenomenon is credited[1] to Mügge (1909) who used autoradiography to disclose the distribution of radioactivity in minerals. It was twenty-five to thirty years before biological use of this method was stimulated by the work of Lacessagne, Hevesy, Hamilton and others.[3, 4, 5, 6]

In the technic for producing autoradiographs as used by Hamilton,[6] thin sections of tissues which contain radioactive material are placed against a photographic film. After sufficient exposure of the film in the dark room, it is separated from the tissue and developed. The tissue section is then stained and it is possible to examine corresponding areas of the tissue and the film, and determine the location and amount of deposition of radioactive element in the tissue by the intensity of darkening in the film.

Several recent modifications in the autoradiographic technic have been developed in order to better localize the radioactivity under high magnification. In one method[7] thin slices of paraffin-embedded tissue are mounted directly on the photographic plate and, after exposure and development of the latter, the tissue is stained with hematoxylin and eosin. The whole preparation is mounted in clarite and covered. The silver granules in the film can be readily localized in the tissue since both are in alignment. A quantitative method of analysis has been developed[8] for alpha-emitting substances by the use of a special alpha-sensitive photographic emulsion. The actual tracks of the alpha particles can be traced in the emulsion and it can be determined whether they originated from the nucleus or the cytoplasm of a given cell. Bélanger and Leblond[9] have succeeded in producing some excellent results by spreading the photographic emulsion directly on the tissue; however, impregnation by the emulsion produces some difficulty in carrying out the histotechnical procedures.

PURPOSE AND SCOPE

This is a report of the distribution and localization of radioactive gallium (Ga^{72}) in the teeth and jaws of experimental animals as revealed by autoradiographs. An analytical study of the tissues of gallium-treated experimental

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Received for publication, April 16, 1949.

animals[10] revealed a marked predilection of this element for calcified structures and led to this study of its distribution in their teeth and jaws. It is believed that information on the metabolism and distribution of certain of the less common elements should be added to the abundant accumulation of data on the more readily available radioactive elements, such as phosphorus,[11-22] sodium,[23] calcium,[24, 25] and fluorine.[26-28] With a complete picture of the activity of all of the elements, it is to be hoped that practical applications and additional therapeutic uses will be forthcoming.

It is not the purpose of this report to present the advantages of using radioactive isotopes in studying the metabolism and distribution of the various elements nor to review the progress which has been made in this field. There have been some detailed accounts of this type of research in the fields of medicine,[5, 6 29, 30] dentistry,[31, 32] chemistry,[33] biology,[34] and physics[1, 34] by a number of competent reviewers.

MATERIALS

Gallium is classed with other rare earth metals[35] (cerium, beryllium, yttrium, and zirconium) and is said to resemble aluminum in chemical and pharmacological properties. It is bluish or grayish-white,[36] has an octohedric crystalling structure, and melts at 30° C. to a mercury-like liquid. It was discovered in 1875 by Lecoq de Boisbaudran as a component of zinc blende. There have been but a few commercial uses for gallium, such as for brightening optical mirrors, for use in vacuum lamps instead of mercury, and use in quartz thermometers. The toxicity of gallium is low, however, considerable doses (100 to 180 mg./kg.) have been found to cause neurological symptoms, blindness, and paralysis experimentally in rats.[37]

For these experiments a water-soluble gallium lactate was used,[38] which was adjusted to a pH of 7.0 to 7.4 by the addition of dilute sodium hydroxide. The radioactive isotope used (Ga^{72}) has a half life of 14.1 hours and is an emitter of beta and gamma rays.

METHOD

1. White rabbits and white rats received 0.2 to 0.4 millicuries of Ga^{72} per kilogram of body weight, injected subcutaneously as the lactate. With each such portion of radioactive gallium, some stable gallium was injected because it is inseparable (chemically referred to as "carrier gallium"), so that an estimated total dosage of 6 mg./kg. of gallium was administered.[39] This quantity is well below the toxic dose.[37] The animals were sacrificed eight hours after the injection and the bones were cleaned of all soft tissue.

2. Two methods were used in sectioning the jaws and teeth: (A) Some of the jaws were embedded in quick-setting dental stone so that they could be held firmly while being cut with a fine-toothed band saw. The cut sections (Fig. 2) were then smoothed on emery paper so that good contact would be made with the photographic film while the autoradiographs were being made (Fig. 1). (B) It was found that better autoradiographs were produced when

the sections were made thin (50 to 100 microns). These sections were made by cutting through unmounted jaws at selected levels with a thin, carborundum-impregnated, hard rubber disc, thereby obtaining intermediate moderately thin sections. Each of these was then cemented to a large cork with acetone-soluble, synthetic resin cement. Mounted sections were then manually ground down to the desired thickness against the side of an abrasive wheel. While still mounted on the cork, sections were placed against the photographic film to produce the autoradiographs (Figs. 4, 6, 8, 10). Later the sections were removed from the cork with acetone and transferred to microscopic slides. Enlarged pictures were made from these slides by transillumination in a standard photographic enlarging camera (Figs. 3, 5, 7, 9).

Fig. 1.

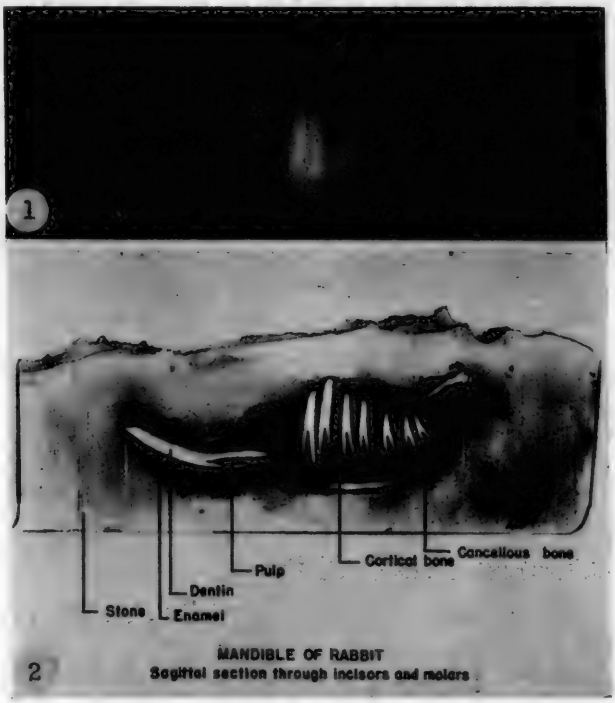

Fig. 2.

Fig. 1.—(mag. × 1.7) An autoradiograph of an embedded rabbit jaw which has been cut sagittally so as to bisect incisor and molars.

Fig. 2.—Drawing of embedded jaw section from which autoradiograph in Fig. 1 was made.

By comparing the two figures it may be seen that pulps of incisor and molar teeth give greatest indication of radioactivity, whereas dentin and enamel give practically no indication of having absorbed any radioactive gallium. Study of the bone shows that cancellous bone has taken up greater quantity of isotope than has dense cortical bone.

3. The exposure time for producing the autoradiographs was 3 to 4 hours for the thick embedded sections and 24 to 48 hours for the thin mounted sections. The film which gave the best results was Eastman single emulsion x-ray film (blue sensitive) No. 6136. Tissue sections which were damp were separated from

Fig. 3.

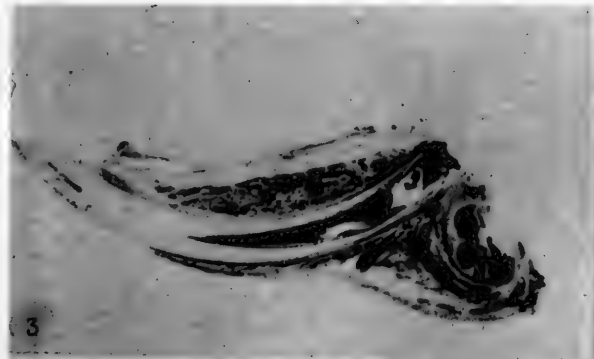

Fig. 4.

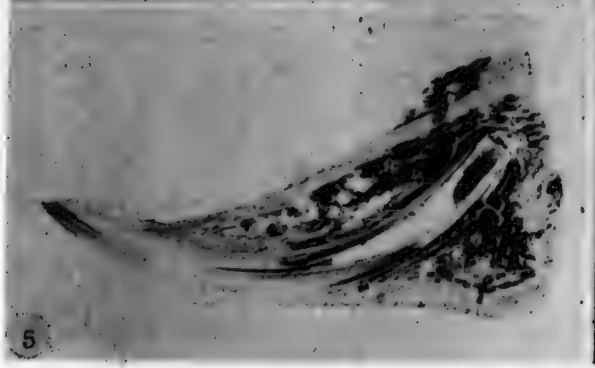

Fig. 5.

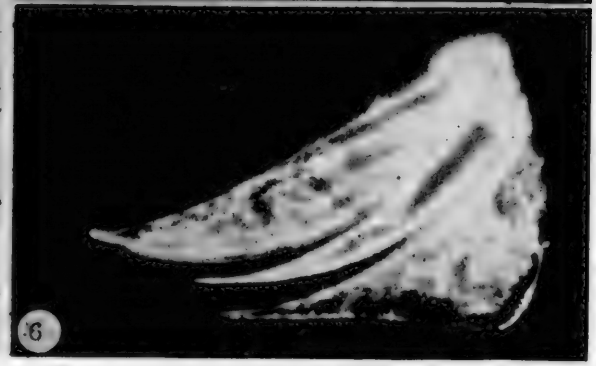

Fig. 6.

Figs. 3-6.—(mag. × 2½-3.) Mandible of rabbit, anterior portion. Sagittal section through incisor.

Autoradiographs (Figs. 4 and 6) made from ground sections of anterior part of rabbit's mandibles (Figs. 3 and 5) show distribution of radiogallium that was injected into rabbits eight hours before they were sacrificed. Intensity of rays emitted from these sections was greatest from periphery of pulps of the teeth, from periosteum, from endosteum, and from periodontal membrane. Cancellous portion of bone exhibited moderate amount of radioactivity. Cortical bone in some areas revealed no evidence of radioactivity, and in other areas it showed slight to moderate activity. Enamel and coronal dentin of incisor teeth show no radioactivity; however, at apex of teeth there is an indication that a trace of radiogallium may have penetrated a short distance into dentin.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

Figs. 7-10.—(mag. × 3.) Maxilla and mandible of rat. Sagittal sections through incisors and molars.

Autoradiographs (Figs. 8 and 10) made from ground sections of rat's maxilla and mandible (Figs. 7 and 9) show distribution of radiogallium which was injected into rats eight hours before they were sacrificed. Localization of radioactive material, as evidenced by effect of emitted rays on photographic emulsion, was greatest in pulps of teeth, periodontium, periosteum, endosteum, and gnarled cancellous bone supporting molar teeth. There was moderate intensity of rays emitted from other portions of cancellous bone, but only slight indication of radioactivity from dense cortical bone. Enamel and coronal dentin of incisors gave no indication of having absorbed any radioactive material. Interpretation of autoradiographs in vicinity of pulps of the teeth, particularly in molar teeth of maxilla, is difficult. By direct examination of slide from which Fig. 7 was made, it is believed that radioactivity arising from these molar teeth was from pulpal tissue. However, it is possible that dentin near the pulp was active. Radiation scattering effects are explained in text.

Several small fragments were lost in mounting section shown in Fig. 9.

the film by paraffined paper or aluminum foil (.025 mm.); however, most of the ground sections were dry enough that they could be placed in contact with the film. The sections were bound tightly to the film with Scotch tape and the whole wrapped in black paper to exclude light. After exposure, the film was developed for six minutes at 20° centigrade.

RESULTS

In this study no attempt was made to determine the radiation dosages qantitatively, even though such measurements are technically possible if standards for comparison are set up by making exposures from calibrated sources.[40] A relative estimate of radioactivity was made by comparing the degree of darkening in several areas of the film. This is in accordance with the principle that the intensity of rays emitted from a certain source will be proportional to the amount of radioactive material contained. The amount of radiogallium absorbed by each portion of the tissue section may be expected to be directly proportional to the degree of darkening in the autoradiograph.

From a study of these autoradiographs it appears that the distribution of radiogallium was essentially the same in each animal, both among the rats and rabbits. The tissues which had the greatest affinity for radiogallium were the periosteum and endosteum of the bone, and the pulp and periodontal membrane of the teeth.* The gnarled cancellous bone supporting the molar teeth of the rats absorbed more of the radioactive compound than the rest of the cancellous bone, whereas the dense cortical bone absorbed only a slight amount. The enamel and dentin of the teeth did not appear to absorb gallium, with the possible exception of the apical dentin which was in direct contact with the pulp. Interpretation is difficult along any fine line which separates two types of tissues because of the fact that rays are emitted radially from any given point. Since the sections had a thickness of 50 to 100 μ, the autoradiographic image of the border between the pulp and dentin of the teeth may have had an overlapping effect of several hundred microns, depending on the range of the radiation particles. For this reason it is most probable that the apparent fringe of radioactivity in that portion of dentin bordering the pulp was actually from the pulp, due to the radial distribution and scattering of radiation particles.

DISCUSSION

The distribution and localization of gallium, as indicated by this study, are shown to be quite different from the distribution of the other elements which have been studied by means of their radioactive isotopes. This is not surprising, however, when it is considered that gallium is not a normal constituent of either teeth or bone, whereas phosphorus, sodium, calcium, and even fluorine are essential to normal metabolism in these structures. The fact that radiogallium is taken up predominantly by bone may make it useful in the treatment of bone malignancies. Its relative absence from the calcified portion of

*The emission of rays from these structures produces the interesting result in the autoradiograph of causing the remaining relatively radioinactive structures to be outlined in white.

teeth only tends to eliminate gallium as a factor in the metabolism of adult tooth structure, even when it is injected in sufficient quantities to be found in the dental pulp.

SUMMARY

Radiogallium in the form of soluble gallium lactate (pH 7.0 to 7.4) was injected subcutaneously into rats and rabbits. The dosage was 0.2 to 0.4 millicuries of Ga^{72} per kilogram of body weight (6 mg./kg.), which is well below the toxic level. The animals were killed in six hours and thin sections (50 to 100 μ) were made of the bone and teeth by cutting with high speed abrasive discs and grinding to the desired thickness against an abrasive wheel. These thin sections were placed next to special x-ray film for 24 to 48 hours. The rays emanating from the radioactive isotope affected the film just as do the rays from an x-ray tube. The developed negatives were compared with transillumination photographs which were subsequently made from the ground sections. By this means it was determined that radiogallium is predominantly deposited in endosteum, periosteum, tooth pulp, and peridontium. There is a moderate deposition in cancellous bone. Cortical bone and the dentin of the root portion of teeth take a barely perceptible amount of radiogallium, whereas coronal dentin and enamel give no indication that any radiogallium is deposited. The main advantages of this method over those using the electroscope or Geiger counter for determining the radioactivity are that fractional portions of teeth and bone are not required, and the visual localization is rapid and effective. The relative distribution of the radioactive substance in each anatomic area is apparent; however, accurate quantitative measurements were not attempted in this study.

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THE RELATIONSHIP BETWEEN PHYSICAL CHARACTERISTICS OF THE DIET AND THE WATER CONTENT OF MONKEY TEETH

N. A. DI SALVO, D.D.S., AND H. H. NEUMANN, M.D.

Dept. of Physiology, College of Physicians and Surgeons, Columbia University, New York, N. Y.

THE following work was designed in an effort to investigate the possible relationship between the degree of masticatory function and certain properties of the teeth which was suggested in preliminary experiments performed on rats.[1] It has been suggested that diminished function of the masticatory structures may be associated with structural changes in the teeth and with a concurrent increase in their susceptibility to caries.[2]

Landoff[3] has shown that bones react to disuse with atrophy which results in a decreased over-all weight of the bone and an increase in the water content. In that study, Landoff pointed out the limitations of histological and radiological technics in scrutinizing these changes. Moreover, it was found that the calcium content per unit of dry weight remained fairly constant.

This last finding is of considerable importance for the bulk of dental research, which has drawn the conclusion that the teeth, once formed, are relatively inert and do not undergo metabolic changes, has been based on data which shows that the proportion of inorganic salts remains remarkably constant. However it has been found that the water content of sound tissues from carious teeth is greater than the water content of noncarious teeth.[4]

Since teeth vary in size from one individual to the next, it was considered inadvisable to compare absolute weights in a relatively small group of animals. The following study is one of water content per unit of weight.

EXPERIMENTAL PROCEDURE

Ten Rhesus monkeys *(Macaca rhesus)*, ranging in age from 37 to 42 months, judging by their dentition,[5] were employed for the experiment. Their body weights ranged from 2.75 to 3.50 kilograms, averaging 3.25 kilograms. Five animals (A, B, C, D, and E in Table I) were kept as controls; the other five being designated as experimental animals (F, G, H, I, and J). They were individually quartered in all-metal cages 36 cu. ft. in size. All animals received ad lib a diet consisting mainly of leafy vegetables, potatoes, bread, bananas, and oranges; however, these items were differently prepared for the two groups.

The experimental animals received their rations overcooked and in a soft, semiliquid consistency so that little or no mastication would be required. Potatoes and other vegetables were well boiled and bread given crustless and water soaked. The control animals received their foods raw whenever possible. In addition, this group received daily raw whole wheat grain and sugar cane; these latter items especially being calculated to require vigorous mastication before

Received for publication, March 31, 1949.

being swallowed. This group was observed from time to time during feedings and it was evident that these items in particular did require forcible and prolonged chewing action.

One week after the start of the experiment, one of the experimental animals (J) succumbed to an acute infection. The teeth were removed post mortem and stored in distilled water to be considered with the others at the close of the experiment. The rest of the animals seemed to fare well on their diets as they showed normal activity and looked well nourished.

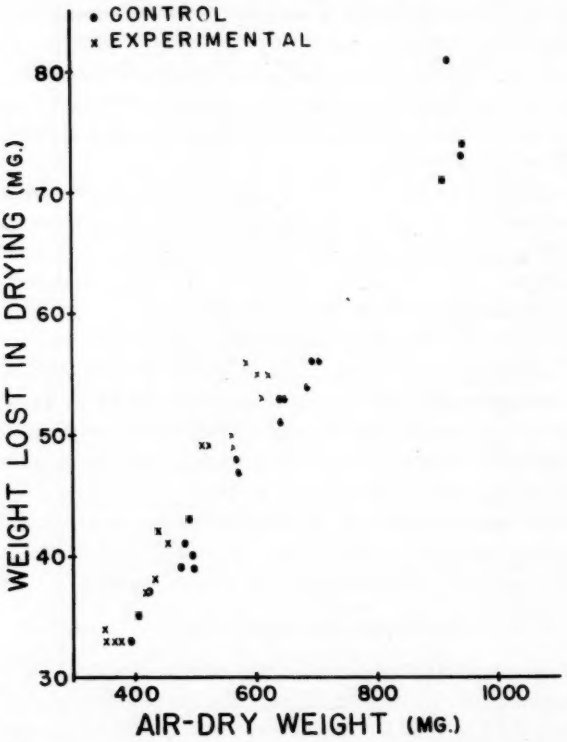

Fig. 1.—The relationship between weight lost in drying and the "air-day" weight.

After eight weeks, each monkey was anesthetized with Nembutal intraperitoneally (0.6 mg./kg.) and the four first permanent molars were surgically removed. The teeth were freed of all adherent soft tissue and were allowed to remain in room air for a period of twelve hours (sufficiently long to obtain a relatively constant "moist" weight) after which each was weighed on an analytical balance.

Having obtained this moist or more correctly "air-dry" weight, the teeth were dried to constant weight. This was accomplished by placing them in a drying oven at 100° C. for 72 hours, after which time they were removed, cooled in a desiccator over calcium chloride, and again weighed.

RESULTS

The loss of weight was plotted against the air-dry weight (Fig. 1) and it was apparent that a linear relationship existed for both groups. However the experimental points were displaced upwards from the control data. By the method of mean least squares, the slope of the line for the experimental group was computed as 0.09 and for the controls as 0.08.

The air-dry weight of the control series varied from 392 milligrams to 952 milligrams with a mean of 630 milligrams. For the experimental group the range was from 351 milligrams to 624 milligrams with a mean of 488 milligrams. It is apparent that the latter group is significantly lighter.

To rule out the weight variable the weight loss was computed as a percentage:

$$\frac{(\text{air-dry wt.} - \text{oven-dry wt.}) \times 100}{\text{air-dry wt.}}$$

The data shown in Table I indicate that the average per cent weight loss due to drying for the control group was 8.17 per cent ± 0.34 whereas the experimental group lost 9.05 per cent ± 0.31. Statistical analysis indicates that the differences are significant.

TABLE I

A COMPARISON OF THE WEIGHT LOSS DUE TO DRYING OF FORTY PERMANENT MOLARS FROM TEN RHESUS MONKEYS

CONTROLS						EXPERIMENTALS					
ANIMAL	TOOTH NUMBER*	AIR-DRY WT. (MG.)	OVEN-DRY WT. (MG.)	WT. LOSS (MG.)	% WT. LOSS	ANIMAL	TOOTH NUMBER	AIR-DRY WT. (MG.)	OVEN-DRY WT. (MG.)	WT. LOSS (MG.)	% WT. LOSS
	1	650	597	53	8.15		21	456	415	41	9.00
	2	688	634	54	7.85		22	440	398	42	9.05
A	3	648	595	53	8.18	F	23	374	341	33	8.82
	4	647	596	51	7.88		24	368	335	33	8.97
	5	949	876	73	7.69		25	608	553	55	9.05
	6	952	878	74	7.78		26	589	533	56	9.52
B	7	928	847	81	8.73	G	27	526	477	49	9.31
	8	915	844	71	7.76		28	518	469	49	9.46
	9	498	458	40	8.03		29	624	569	55	8.83
	10	479	440	39	8.14		30	611	558	53	8.68
C	11	392	359	33	8.42	H	31	562	512	50	8.90
	12	407	372	35	8.60		32	566	517	49	8.67
	13	709	653	56	7.90		33	424	387	37	8.72
	14	699	643	56	8.02		34	434	396	38	8.75
D	15	572	524	48	8.40	I	35	353	319	34	9.64
	16	425	388	37	8.71		36	351	318	33	9.40
	17	573	526	47	8.20		37	417	384	33	7.93
	18	500	461	39	7.80		38	527	487	40	7.60
E	19	483	442	41	8.48	J†	39	426	392	34	7.99
	20	491	448	43	8.76		40	429	395	34	7.93
Averages		630	579	51	8.17			488	444	44	9.05
Average deviation		179	166	14	0.34			96	88	8	0.31
Standard error		40	37	0.7	0.08			24	22	0.5	0.08

*Teeth Nos. 1, 2, 3, and 4 are the four first permanent molars from the same animal arranged in the following order: Upper right, upper left, lower right, lower left. This order follows throughout the paper.

†Animal J died prematurely; these teeth are not included in the averages.

From the data in Table I it appears that the molars of the animals on an entirely soft diet had a water content 10.77 per cent higher than the molars of the vigorously chewing animals.

SUMMARY

Ten Rhesus monkeys were divided into two groups of five each. Both groups received essentially the same diet, except that the physical consistency of the food was altered in the preparation so that the control group received food requiring vigorous mastication and the experimental group received food requiring little or no mastication. The control group received in addition raw whole wheat grain and sugar cane to insure prolonged and forceful chewing.

After eight weeks, the four first permanent molars were removed from each animal, cleaned and weighed after drying in air for 12 hours: subsequently the teeth were dried to constant weight and reweighed. The weight loss was then calculated as a percent of the initial weight.

The control teeth lost an average of 8.17 per cent, the experimentals an average of 9.05 per cent, that is, the molars of the animals on an entirely soft diet had a water content 10.77 per cent higher than the molars of the vigorously chewing animals.

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